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Chemical Regulation of Sexual Processes in the Thallophytes ..... JOHN R. RAPER 447

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THE BOTANICAL REVIEW

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CHEMICAL REGULATION OF SEXUAL PROCESSES IN THE THALLOPHYTES*

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INTRODUCTION

The hypothesis that the differentiation, development and proper function of vegetative and sexual organs of plants depend upon specific chemical substances was advanced by Sachs (158) more than 70 years ago. Surprisingly little information of the physiological details of the sexual reproductive process has been brought to light during the intervening years, and the actual mechanism of sexuality in plants in general is but slightly better understood at the present time. The initiation and differentiation of sexual reproductive structures, as well as their subsequent activities in the reproductive cycle, can be profoundly affected by the manipulation of chemical and physical factors of the plant's environment, but in only rare instances is there more than a very superficial understanding of the means by which these effects are achieved. When the present state of knowledge of the endocrine system in mammals

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or the correlative mechanism in growth phenomena in higher plants is contrasted with that of the sexual process in plants, a field of comparable biological interest, the realization is inescapable that here is a relatively neglected major field of biological study. That this should be the case is indeed surprising in view of the far more exact control over fruit and seed production by crop plants which would almost certainly result from more detailed knowledge of the mechanisms involved in the sexual processes of higher plants.

Studies with higher plants designed to elucidate the chemical control of sexuality (42), while indicating that such control is operative, have for the most part yielded but scant results pertaining to the details of the process. The cause for this possibly or even probably lies in a combination of factors, including the chemical and physiological complexity of the control mechanism, undesirable chemical properties of the compound(s) initiating the reaction chain, and the failure, a natural consequence of the first two factors, to develop as yet the techniques and concepts necessary for the solution of an exceedingly intricate problem.

Greater advances have been made along these lines of study in the more primitive groups of plants, particularly in the Thallophytes. Perhaps this is a result solely of the more advantageous habits of growth and morphology of these plants which, in large part, are more intimately associated with their environment and hence are more rapidly responsive to minute changes in their environment than are the higher plants. While there is available no basis for such an assumption, it is possible that the physiological and morphological changes involved in sexual reproduction in the "simpler" plants, the fungi and algae, are less complicated and more subject to external control than comparable processes in the more highly evolved flowering plants. Be that as it may, there are numerous papers extending throughout the past half-century which deal in whole or in part with observations on the alteration or control of sexual processes by experimental means. Many of these observations have resulted from chance discoveries and most have dealt with isolated cases in single species where there were no attempts made to elucidate the mechanisms of the effects described. Recently there have been a few studies which, being pursued over a period of years and involving either several related species and/or an exhaustive study of biochemical systems, have postulated de-

tailed and integrated mechanisms for the chemical regulation of certain portions of the sexual process. Comparison of the result of these studies, which involve unrelated organisms and even different restricted sequences within the sexual process, serves to point out striking differences instead of indicating a common mechanism of sexual regulation, a conclusion in no way affected by a consideration of the more voluminous but fragmentary data furnished by casual observations extending over half a century. Whether it is indeed the case that there exist many different regulatory mechanisms, perhaps as many as there are morphologically different sexual apparatuses?, or that there is a basic mechanism common to all plants, and that the variations from group to group are only differences in detail, is a moot question, answerable only at some time in the future when sufficient data have been amassed.

It is the purpose of the present paper to review in considerable detail the accumulated observations dealing with all aspects of the chemical regulation of sexual processes in the various groups of plants comprising the Thallophytes. These observations fall naturally into two sharply divided categories: *a*) those effects which can be measured only in terms of the quantity or quality of the final product of the entire sexual process, i.e., substances of known or unknown origin and nature which affect for any given conditions the relative number of oospores, zygospores, asci, basidia, etc.; *b*) those effects which can be assigned to definite and limited phases of the sexual process and are demonstrably due to specific substances of known origin, i.e., the formation, response or development of specific organs or sexual cells under the stimulus of chemical agents produced in most cases by the same plant or other plants of the same species. The papers comprising category *a* will be considered roughly in chronological order, while those of category *b* will be examined in connection with the specific sexual phases with which they deal in an attempt to integrate so far as is possible the various and often superficially dissimilar sexual mechanisms of the several groups of fungi and algae.

A third and less distinct category is composed of observations and speculations, unsupported by definitive experimental evidence, which attempt on rational grounds an explanation of well-known phenomena dealing with specific sexual reactions. These will for the most part be distributed among the two former categories as

best suits each individual case; in a few cases, however, there are instances of extremely common reactions, for which a search of the literature has yielded neither experimental nor even speculative explanations. Some of the more obvious of these cases will be dealt with in a totally speculative manner to demonstrate at least the feasibility of interaction based on chemical regulation.

The terminology used in this review is of necessity rather arbitrary. Two distinct types of reactions are involved: *a*) a definite influence on sexual activity, either a stimulation or a suppression of the entire sexual process or of restricted phases thereof, brought about by the presence of a specific substance or group of substances originating external to the affected plant; these substances, because of their extra-species origin and their hormone-like activities, will be termed " ectohormoids "; and *b*) the regulation of sexual processes by substances produced by the affected plant or by other individuals of the same species; these chemical substances of intra-species origin, performing indispensable regulatory roles in the sexual process, will be termed " hormones ".

This use of the term " hormone " perhaps demands certain justification, since its use in this connotation departs widely from the original definition of the term. Hormones were first defined by Bayliss and Starling (10) as substances which, being produced in one part of the animal body, are carried by the blood stream to another part where they bring about a specific physiological reaction. Went and Thimann (181), in their monograph on growth regulators of the higher plants, revised this definition to include the auxins. They define a hormone as a chemical substance which, being produced in one part of an organism, is transported by any means to another part where it elicits a specific reaction. The emphasis on transportation and its mechanical means of accomplishment as a criterion of definition was criticised by Huxley (55) who emphasised, instead, the role which the chemical entity performs in the vital activities of the organism. His classification separates hormones into two groups: " regional activators ", produced in and eliciting their specific responses within restricted regions, and " distance activators ", produced in one portion of the organism and transported by any means, including diffusion, to other portions of the same individual or to other individuals of the same species where they induce specific responses. It is according

to this interpretation and system of classification that the term " hormone " will be used in the present paper.

Extra-Specific Chemical Regulation

The published examples of extra-specific regulation of sexual processes in algae and fungi, reported for the most part as observations incidental to other studies, constitute a rather ill defined body of information. The subject as interpreted here will include all those cases in which agents of chemical nature, or agents of physical nature which appear to operate through chemical intermediaries, specifically affect sexual reactions. The presentation of these cases departs from a chronological order only in a few instances in which numerous papers on a single subject, appearing over an extended period, have necessitated a certain amount of subject integration.

The relationship between nutrition, vegetative growth and vigor, and sexual reproductive processes in algae and fungi received much intensive work about the turn of the century and continues to be a subject of much interest. The voluminous work of Klebs (59, 60, 61, etc.) served as the prototype for that of several workers in America, such as Kaufmann (57), Pieters (132) and Coker (24), all of whom worked chiefly with members of the aquatic phycomycetous family, the Saprolegniaceae. The general pattern of relationship between nutritional availability, vegetative characteristics and qualitative and quantitative sexual expression was early established by Klebs and abundantly confirmed by subsequent investigators (summary and review in Coker (24)). While there were many variations between different species, the following generalizations were unquestionably established:

1. Cessation of continuous and indeterminate vegetative growth depends only upon the exhaustion of suitable nutrient or upon a general staling of the medium.

2. Sexual and asexual reproductive organs are formed when a well-nourished mycelium exhausts its available food supply or is suddenly deprived of nutrient by transfer to water.

3. By proper choice of nutrient materials, furnished in the proper concentration, and by varying environmental conditions it is possible to influence the developmental history of the plant to a tremendous degree: *a*) growth with continuous production of zoosporangia but

lacking oogonia; *b*) limited growth with active production of oogonia; *c*) active vegetative growth followed by formation of oogonia, in certain species with or without antheridia, depending on the whim of the investigator; *d*) active growth followed by formation of zoosporangia and later by oogonia; *e*) growth with concurrent production of oogonia and zoosporangia; *f*) active growth followed by production of oogonia and later by zoosporangia; etc. The quantitative variations which may be induced in the relative number of oogonia and antheridia, size of oogonia and oospores, percentage of oospore maturation, etc. are myriad.

4. The mycelium, during a period of growth in a given nutrient medium, may be so affected as to influence the characteristics manifested at a later time following transfer to a new environment.

5. The presence and concentration of certain inorganic salts may exert a profound influence either on specific stages of the sexual process or upon the over-all efficiency of the entire reproductive process (57).

These studies were made for the most part with unifungal, but not with sterile, cultures, and they are consequently open to the criticism that certain of the described effects may have resulted from the metabolic activities of contaminating mocroorganisms. The reproducibility of cause-effect relationships and the agreement between different workers would seem, to a great extent, to mitigate such objections.

The first of a large number of papers, continuing to the present time, dealing with the effects of sexual ectohormoids, i.e., effects of the metabolic products of microorganisms upon the reproductive processes of other species, usually of widely different groups, appeared in 1903 with the observation of Molliard (120) that the number of apothecia produced by a species of *Ascobolus* was greatly influenced by the presence or absence of certain contaminating bacteria.

Of the early works dealing with such inter-species effects, that of Heald and Pool in 1909 (52) is outstanding. These workers showed that single ascospore cultures of *Melanospora pampeana* produced but few conidia and no perithecia in pure culture. Both conidia and perithecia, however, were produced in profusion by this species when grown in mixed culture with certain species of *Fusarium* and with *Basisporium gallarium*, although neither type

of reproductive body was produced in mixed cultures with other species of *Fusarium* or with other fungi tested. Filtrates of cultures of *F. moniliforme* and *B. gallarium,* aged two, three, four and six weeks, were sterilized at 110° C and inoculated with sterile mycelia of *Melanospora.* In the filtrates derived from the old cultures no perithecia were induced; perithecia were produced in abundance in the filtrates of the young cultures. It was observed that the active filtrate was acidic, and preliminary chemical fractionation showed the effect to be due to a complex organic acid. It was also shown that previous or concurrent growth of *Pilobolus* on horse dung was a necessary condition for the fruiting of *Sordaria longicaudata.*

In connection with a study of the morphology of the archicarp in species of the Ascobolaceae, Dodge, in 1912 (31), made some interesting observations on the fruiting habits in this family. Normally, apothecia are first formed around the edge of the culture plate shortly after the time at which this point is reached by the growing mycelium, and only later are they produced at points scattered over the entire mycelium. When inoculations are made at two points at opposite sides of the plate, a restraint area is formed along the line of contact and apothecia appear soon thereafter on each side of this area. It was further observed that some bacterial contaminants stimulated fruiting significantly in the vicinity of the colonies and that certain of these produced a region of vegetative inhibition around them.

In the same year the French worker Sartory (159) published the first of three papers dealing with comparable inter-specific effects. A yeast isolated from banana would not fruit under any of a large number of conditions tried; when it was associated in a two-membered culture with a bacterium with which it was originally growing upon the banana, it fruited both readily and copiously. Furthermore, sporulation of the yeast in the presence of the bacterium was shown to be a function of temperature, occurring only within the range 15–22° C.

In later papers (Sartory, 160, 161) two species of *Aspergillus,* isolated from feathers and bananas, respectively, were shown to produce perithecia only under the influence of two accompanying species of bacteria: one was a member of the *B. mesentericus* group, the other was not identified. In both cases, a number of

known species of bacteria, including *B. subtilis* and *E. coli,* gave negative results. The author, in seeking to explain these effects, postulated that in each case the bacterium effected changes in the chemical composition of the medium which resulted in the production of fruiting bodies.

The inter-specific chemical stimulation of sexual processes in *Thielavia basicola* was reported in 1925 by McCormick (93), who demonstrated the effectiveness of extracts of other fungi in the production of perithecia in this species.

In the following year an exhaustive study by Robinson (156), dealing with conditions of growth and development in *Pyronema confluens,* showed a remarkable dependence of sexual reproduction on the constituents and physical conditions of the medium and the environment. The C/N ratio was shown within wide limits to be of less importance in reproduction than the absolute concentration of each of the two elements above a minimal level. Low concentration of nutrient would suffice for reproduction, and the reproductive processes were shown to be initiated only upon exhaustion of the NO_3^- ion. In vigorously growing mycelia no reproductive structures arose, but they could be induced at will either by removing the unused peripheral agar medium or by introduction of certain chemicals. A number of inorganic salts, including $AgNO_3$, LiCl and $K_2Cr_2O_7$, used as local poisons on the periphery of the vegetative mycelium, induced apothecial formation shortly after their application. A further interesting series of observations dealt with the role of light and its relation to specific pigments in the reproductive process. Light in the blue portion of the visible spectrum is required for reproduction but not for growth; in the dark or in red light only whitish wisps of hyphae, which change to black on aging, are formed. The effect of light was shown to be quantitative, and a small quantity suffices for normal development. Very short exposure to light allows formation of antheridia and ascogonia, while, at the same intensity, longer exposure is required for the continued development leading to formation of normal spore-bearing apothecia. A pink pigment is always associated with mycelia which are reproducing normally, while it is absent from mycelia kept in the dark or exposed to conditions which prevent reproduction. Carbon dioxide in concentration of 1–5% effectively inhibits both pigment production and reproduction. The role of

this pigment was not determined, but it would appear to be intimately related to the sexual process.

A series of investigations by Satina and Blakeslee, the first publication of which appeared in 1926, was undertaken in an attempt to discover biochemical criteria of sexual differentiation in the heterothallic species of the Mucorales and to compare such differences in these forms with those existing in other groups of plants and animals. The results embodied in this and in subsequent papers of this series (162, 163, 164), while lacking the decisiveness hoped for by the investigators, contain much of interest to those who would attempt to understand the underlying mechanism of sexual reproduction in any of the microorganisms. The following examples serve to illustrate the method of approach as well as the intrinsic difficulties of the work: *a*) races of *Mucor* differ in biochemical characteristics, even when belonging to the same species and sexual strain; *b*) sexual strains show no differences as regards internal hydrogen-ion concentration; *c*) (−) and (+) strains show significant average differences as regards a number of biochemical characteristics, including the Manilov reaction, catalase content, reduction of $KMnO_4$ by cell-free extracts and reduction of tellurium salts by the living cells, etc.; *d*) sexual strains show probable differences in peroxidase content and total acid production. These results, when compared to those of the same tests applied to male and female individuals of a number of other plants and animals, led the investigators to consider the (+) strain as female and the (−) strain as male. On the basis of the results obtained, a high degree of accuracy could be achieved in predicting the mating reaction of an isolate of a heterothallic mucor prior to mating it with known sexual strains.

The pattern of sexuality and the conditions necessary for sexual reproduction in various species and strains of *Phytophthora* have been the subject of intensive work beginning with that of Gadd in 1925 (38). Oogonia and oospores were found in certain matings between strains of *P. faberi* but not in others, and the species was therefore considered to be heterothallic. Lester-Smith (90), however, considered that each strain was weakly self-fertile and that enhanced sexual vigor in matings resulted from lowered metabolic activity due to interstrain competition. It was conclusively shown that such alleged weakly self-fertile strains would produce oospores

abundantly when mated with *P. parasitica,* a species universally accepted as homothallic. With the precedent of Blakeslee's clear demonstration of the failure of inter-specific matings in the Mucorales (17), this was cited as proof of homothallism, the expression of which depended on chemical changes induced in the medium by another strain! A series of papers by Ashby (4–7) failed to clarify the problem of sexuality in *Phytophthora.* He showed that in various species oogonia and oospores appeared promptly in inter-strain matings or did not appear at all; he also confirmed the earlier observations on the reaction of apparently self-sterile strains when mated with *P. parasitica.* Finally, the discovery of oogonia and oospores in old cultures of strains previously considered as self-sterile resulted in Ashby's denial of heterothallism in the genus and his acceptance of the hypothesis of sexual expression through chemical stimulation.

Heterothallism in *P. omnivora* was definitely established by Leonian in 1931 (85). By inter-strain matings of some 85 isolates from different locations he showed that four distinct groups, based on sexual behavior, could be distinguished: *a*) homothallic, *b*) heterothallic, *c*) heterothallic but quantitatively inconstant, and *d*) neuter. The second group, the heterothallic isolates, comprised approximately one half of all the isolates. A similar pattern of sexuality is now known to be relatively common in all groups of the higher biflagellate aquatic Phycomycetes (15, 20, 21, 28, 134, 138).

The constituents of the medium were shown to have great importance in the sexual process. Vigorous production of oogonia occurred on a medium containing oatmeal, while no oogonia were formed on malt extract medium. Another species, *P. cactorum,* would not grow on a synthetic medium containing dextrose, whereas addition of a small quantity of an alcoholic extract of canned peas resulted in luxurious growth and abundant oogonia (86, 87). Introducing an uninjured tip of corn root into the culture had the same effect. Addition of proteose-peptone to the synthetic medium permitted vigorous vegetative growth of the mycelium without production of any oogonia. Activity comparable to that of pea extract in stimulating the production and development of sexual organs was contained in the alcoholic extracts of certain unicellular green algae. When plants grown in the minimal me-

dium, supplemented by proteose-peptone, were transferred to media containing extracts of *Coccomyxa* or *Oocystis,* oogonia were formed in abundance; if transferred to media containing extracts of *Chlorella* or *Scenedesmus,* few oogonia were produced; if transferred to fresh medium of the same composition or to water, no oogonia were produced. The alcoholic extract of *Oocystis* cells, was shown to be rich in substances which enhanced growth and induced sexuality whereas ether extract contained only the sexually stimulating substances. The substances extracted from the algae which promoted sexual activity were termed "auxins" by the author without any clear evidence of their identity with the growth substances of higher plants.

The number and size of perithecia of *Venturia inequalis* were shown by Wilson, in 1927 (182), to be strongly influenced by the secretions of a species of *Penicillium.* Sterile cultures of *V. inequalis* were grown on Petri plates, and the mature mycelia were flooded with *a*) water, *b*) raw filtration-sterilized brei of *Penicillium,* and *c*) autoclaved brei of *Penicillium.* The number and average size of the perithecia observed in forty 2.5 mm.^{2} areas for these three treatments were respectively: *a*) 2, 37 μ; *b*) 25, 67 μ; *c*) 6, 49 μ.

Schopfer, in 1928 (165), and Ronsdorf, in 1931 (157), demonstrated the importance of the C/N ratio in the sexual vigor of *Mucor hiemalis* and *Phycomyces blakesleeanus,* respectively. Both of these forms were shown to require carbon and nitrogen in specific minimal quantities as well as in a definite ratio for normal zygosporic formation. The latter author tested the effects of a number of compounds on zygosporic formation, of which two are of considerable interest. Phloroglucin was shown to suppress zygosporic production completely without affecting vegetative growth, and histamine, in concentration less than 10^{-6}, was shown to induce "immediate" production of zygospores.

A case closely resembling the activities of sexual ectohormoids has been reported in the Fungi Imperfecti and is of passing interest. Two saltant strains of *Cystospora ludebunde,* each individually infertile, were shown by Das Gupta (29) to produce pycnidia when grown together. The pycnidia of the two strains were characteristically different, and it could thereby be shown that only one strain contributed to the formation of each pycnidium. This was

held as evidence that a pseudosexual union, with the establishment of a heterokaryon, was unlikely and that a more probable explanation could be found in the theory of "nutritive heterothallism" earlier suggested by Gwynne-Vaughan and Williamson (40).

During the period 1933–40 a number of papers appeared showing the activity of secretions of various microorganisms in stimulating the sexual processes in other species.

The nutritional and accessory growth requirements for perithecial formation in a number of Ascomycetes was the subject of several papers by Hawker and associates (8, 48, 49). A quantitative study of perithecial production in relation to nutrition in *Melanospora destruens* showed that the C/N ratio is of less importance than the absolute level of available nutrient. Decrease in the concentration of PO_4^{---} decreased both growth and sexual reproduction. Transfer of a mycelium from a full to a weak nutrient induced formation of perithecia. Light, temperature and pH appeared to be of little importance. Perithecial formation in *M. destruens* was markedly influenced by the presence of contaminating fungi, provided that inoculation was so adjusted that neither mycelium overgrew the other. In practically every case the stimulation was quite local, perithecia forming in increased number only at the edge of the contaminating colony. Media "staled" by a large number of fungi, including species of *Botrytis, Fusarium, Gleosporium, Sclerotinia* and *Penicillium,* were shown to shorten the time of appearance and to increase the abundance of fruiting bodies of *M. destruens,* provided the cultures were not too old, in which case an inhibitory effect obtained. The ether-soluble fraction of active "staled" media was shown to be much more effective than either the entire medium or the combined ether-soluble and ether-insoluble fractions. The interplay of three factors was postulated as responsible for perithecial production: *a*) secretion of stimulating substances, *b*) staling products which supress growth, and *c*) level of unused nutrients in the medium.

In the later work (48, 49) it was shown that extract of lentil was effective in increasing perithecial production in proportion to its concentration up to a maximal value. This stimulation could not be induced by the inositol fraction but only by the inositol-free fraction of the lentil extract. The same stimulatory effects were described for a large number of Ascomycetes tested. Either biotin

or thiamin alone added to a synthetic medium would permit growth of *M. destruens,* while both of these accessory substances must be added for fruiting to occur. Certain saltant strains could grow and fruit in the absence of biotin and/or thiamin. Addition of either increased fruiting, while addition of both caused spectacular increase. Increase in thiamin concentration was shown to raise the concentration of glucose required for optimal perithecial formation. Biotin had no such effect. *Podospora curvula, Sordaria fimicola* and *Sordaria* sp. resemble *M. destruens* in their growth and fruiting requirements.

More recently Hawker (50, 51) has shown that phosphorylated hexoses significantly stimulated the production of perithecia in *M. destruens.* Glucose 1-phosphate, fructose 1,6-diphosphate, or a mixture of monophosphates were equally effective. Sucrose or fructose (vs. glucose) gave increased perithecial formation. This was interpreted as the result of the greater ease of phosphorulation of fructose than of glucose.

Hot water extract of lentils was also shown to increase the intensity of the sexual reaction in *Achlya ambisexualis* in matings on agar. In distinction to the findings in the Ascomycetes cited above, i-inositol here is almost certainly the effective agent (138).

During the decade 1932–42 the requirements for growth and zygosporic formation in *Phycomyces blakesleeanus* were the subjects of a large number of publications, chiefly by Schopfer and Robbins and their respective associates. It was first shown in 1931 by Schopfer (166, 167) that thiamin was indispensable for growth of this species. Later results indicated that fruiting depended upon other requirements apparently present in natural substrates but lacking in a synthetic medium. Maltose furnished this unknown requisite, while glucose and gelatin did not. Other sources of the zygospore-forming substance were later reported in corn, oatmeal, potato, agar, etc. (147). Factors other than specific accessory requirements already shown to affect zygosporic production were *a*) distance between points of inoculation of the (+) and (−) strains, the reaction intensity being inversely proportionate to the distance between inoculations; *b*) effect of high N or C content, the zygospore-forming substance in the medium being exhausted in heavy vegetative growth; and *c*) the effect of temperature, the synthesis of the substance being greater or the requirement less at

low (15–20° C) than at high temperatures (25–28° C). The intensity of the reaction, i.e., the number of zygospores produced under standardized mating procedure, was shown to be proportionate to the concentration of the agar: no progametangia at 1%, a yellowish line of gametangia with a few zygospores maturing at 2%, and a much heavier line of gametangia with as high as 62 zygospores maturing at 3% agar content. The active substance could be extracted from agar by methyl alcohol or washed out quantitatively by running water (148).

Potato extract (152) was shown to contain an active substance(s) affecting spore germination, vegetative growth and zygosporic production; in each case the effect was proportionate to the concentration of the extract in the medium. Fractionation of the potato extract by adsorption on Norite revealed two active principles; one was adsorbed on the Norite, the other remained in the filtrate. These two principles were named factors Z_1 and Z_2, respectively. Each of these factors brought about increases in each of the three developmental stages (germination, growth, zygosporic formation), but neither was fully effective without the other, i.e., greater effect obtained when the two were combined than the sum of the effects of the two used separately. While potato extract is rich in both factors Z_1 and Z_2, other natural products were shown (150) to contain the two factors in varying proportion; for example, neopeptone is rich in Z_1, less rich in Z_2, while Difco agar is rich in Z_1 and contains hardly any Z_2.

The beneficial effects of agar, potato and other natural products were shortly confirmed by Schopfer (170) who designated the required substance or complex as factor M (maltose).

The contention that specific substances, in addition to thiamin, are required for zygosporic formation by *P. blakesleeanus* was disputed by Leonian and Lilly (88). These workers maintained that the C/N ratio was important and that weak sexual expression resulted from improper balance between these elements. They demonstrated that heavy zygosporic formation was possible in a synthetic medium containing thiamin, glucose, any one of several individual amino acids, and inorganic salts. Of the amino acids, aspartic acid was the best. The production of zygospores was benefited by addition of succinic acid. NH_4NO_3, which is not utilizable as a N-source by the fungus, was shown to inhibit sexual

expression completely, while in no way affecting vegetative growth. The inhibitory action of NH_4NO_3 could be partially overcome by addition of succinic acid. Tests with various minor elements revealed no absolute requirements.

That *P. blakesleeanus* can synthesize, perhaps in sub-optimal quantity, any absolute requirement except thiamin was shown (149) by the effect of extracts of vegetative mycelia upon its vegetative and reproductive development. Such extracts, however, were less effective than those of potato and agar. The effects of the extracts increased and then decreased with age of the mycelia from which they were made, the maximal benefit obtaining at the age of 7–11 days.

In 1942 the purine-base guanine (153) was shown to have factor Z_1 activity, and it could be substituted for the factor derived from various natural sources. On the basis of relative adsorption, stability and solubilities it was clear, however, that guanine and factor Z_1 were not identical. Factor Z_1 was also shown not to be identical with any of the known vitamins. Factor Z_1 was identified later the same year as hypoxanthine (154), another purine-base closely related in chemical structure to guanine. Hypoxanthine, both synthetic and isolated from potato, used together with factor Z_2 from potato, greatly increased growth of the fungus. Robbins (151) demonstrated hypoxanthine to be quite specific as factor Z_1.

Further studies by Robbins and Schmitt (155), designed to elucidate the chemical nature of factor Z_2, demonstrated instead that the activity previously ascribed to a specific accessory factor could be duplicated by numerous agents which prevented local accumulation of excessive hydrions. Progametangia were produced in large number whenever vigorously growing (+) and (−) mycelia met in a region in which the hydrogen ion concentration was maintained at or slightly above a critical value of pH 4.00. This could be achieved through addition to the medium of the calcium salts of glutaric acid or a large number of other weak organic acids, by inoculation of the two strains close together or by local neutralization of acidic metabolic products with KOH.

In the later papers, excepting that of Robbins and Schmitt (155), on the accessory substance requirements for growth and zygosporic formation in *P. blakesleeanus* there was progressively less attention paid to zygosporic production and more to the role

of Z_1 in various aspects of vegetative growth as the series continued. It would appear that factor Z_1, originally defined as a specific requirement for sexual expression, turns out to be primarily concerned in vegetative growth, as is thiamine, and that the requirements leading to zygosporic production are manifest by physiological conditions established in the mycelium.

Schizophyllum commune (172) is heterotrophic for thiamin, but even with added thiamin the diplophase (dicaryophase) produced only stunted, weak sporocarps. Addition of a complex natural substrate allowed proper fruiting, and it was concluded that factors other than thiamin were also required for completion of the sexual process.

An interesting example of inter-specific stimulation of a sexual process was discovered and elucidated by Nickerson and Thimann (126, 127) for *Zygosaccharomyces* sp. This species in pure culture showed only 8–12% conjugation, while in cultures contaminated with *Aspergillus niger* the incidence of conjugation was increased to 65–75%. This effect could be readily duplicated by addition to the yeast culture of culture filtrates of the contaminating *Aspergillus* but not by hydrogen-ion adjustments. The active compound(s) was shown to be soluble in 90% ethyl alcohol but not soluble in acetone or chloroform. The active substance(s) could be extracted from the cells of *Zygosaccharomyces,* a finding which was suspected in view of the common, if low percentage, conjugations between cells in pure culture. Interesting correlations between certain physical factors and the reproductive process were described in the second paper: percentage of conjugation in liquid and in agar cultures was about the same, but on agar the average length of the conjugation tubes was much greater; on agar slants there was a significantly higher percentage of conjugation on the thin end of the slant, and this increased sexual activity could be correlated with the greater number of dead cells in this region, as shown by methylene blue staining. The latter fact points to the probable role of diffusible stimulating substances originating from moribund or dead cells in aging cultures in the normal reproductive process of the yeast. The stimulating activity of the filtrate from *A. niger* was shown to be organic and to consist of two fractions, an organic acid and a member of the vitamin B complex. Each alone was active, but together they gave activity greater than

the sum of their single activities. The stimulatory activities of these two fractions could be duplicated by glutaric acid and by riboflavin. Whether these two compounds are identical with the active substances secreted by *A. niger* and *Zygosaccharomyces* was not determined.

The only demonstrated instance of a significant effect upon the sexual process of a fungus by mammalian sex hormones is that described by Plumb and Durrel (133), in which oestrin or theelin was shown to suppress completely the production of gametangia and zygospores in *Rhizopus nigricans.*

Table I, adapted from an earlier review by the author (144), presents a summary list of the demonstrated cases of extra-specific stimulation of sexual processes and of the few cases in which known chemical compounds exert a significant effect upon sexual reactions among the fungi.

There appears to be little indication, among these demonstrated cases of the activity of sexual ectohormoids, that the regulation of sexual processes by such agents has any profound biological significance. The compounds which show activity, so far as their identities have been determined, are either certain of those substances which are required in small amounts by all living cells, not known elsewhere to have specific sexual activities, or substances which have physiological activity in higher organisms. Furthermore, the effects of these substances of external origin are for the most part quite non-specific and are reflected in overall stimulation or inhibition of the entire sexual process instead of specifically affecting single stages of the process. Thus it would appear that their effectiveness upon the sexual productivity of the plant is indirect and perhaps incidental to primary involvement in general metabolic activities. It is also evident that in most cases the presence or the absence of these external factors is not of critical importance in the sexual process; absolute necessity for external supply of specific compounds for normal progression or consummation of the sexual process is certainly the exceptional case. The activity of such commonly occurring compounds as the various members of the vitamin B-complex, hypoxanthine and glutaric acid can most easily be rationalized as the supplementation of sub-optimal synthetic processes of absolute requirements in the affected plant. The other cases, involving two mammalian hormones and

a substance of wide occurrence in higher plants, would appear entirely explicable as physiological accidents. In any event, it appears unlikely that the regulation of sexual processes by chemical agents of external origin has played any decisive role in the evolutionary history of the Thallophytes.

Specific Chemical Regulation

A full appreciation of the significance of the role of intra-specific sexual secretions and their activities in regulating the sexual processes in any particular plant depends primarily on a complete understanding of the developmental history of the species. In comparing the correlative chemical mechanisms of different species, which may belong to widely separated groups, it is often true that no direct comparison can be made because of the lack of absolutely equivalent processes in the two species under consideration. Failure to recognize this has led to the abuse of many terms and concepts, each useful within the restriction of its original definition, through their unjustified application to processes or chemical agents which appear to be comparable but which, considered in the context of the developmental patterns of the two, are fundamentally dissimilar. A case in point is Hartmann's (46) application of the terms " gamones " and " termones " of Moewus (see below) to the sexual secretions in *Achlya* which induce formation of sexual organ primordia. The overlap of the correlative mechanisms in *Chlamydomonas* and *Achlya*, as they are now understood, is indeed slight and there is no reason to assume, a priori, a single over-all pattern of regulation in both. On the other hand, it is equal folly to assume that, since the sexual processes of two species are superficially very dissimilar, there is no equivalence between them.

The primary basis for a discussion and comparison of sexual mechanisms in widely separated plants must, therefore, be a means of determining the exact course of sexual development in each species considered and of integrating each into the broad and complex pattern of sexual phenomena in the entire division of Thallophytes. Sexual processes in all fungi and algae are here divided into four basic types, first recognized and described by Kniep (62). Each type comprises definite groups of plants but has little correlation with the major phylogenetic groupings. The four basic types are:

1. Gametic copulation, in which the two nuclei brought together

TABLE I

EXTRA-SPECIFIC CHEMICAL REGULATION OF SEXUAL PROCESSES IN FUNGI

Species affected	Source	Effects[1]	Identity	Authority
Ascobolus sp.	Bacteria?	Apothecia	?	(120)
Melanospora pampanea	*Fusarium* spp., *Basisporium gallarium*	Perithecia	Organic acid	(52)
Sordaria longicaudata	*Pilobolus* sp.	Perithecia	?	(52)
Ascobolus magnificus	Bacteria?	Apothecia	?	(31)
Yeast?	Bacteria?	Asci	?	(159)
Aspergillus	Bacteria?	Perithecia	?	(160, 161)
Theilavia basicola	Other fungi?	Perithecia	?	(93)
Phytophthora cactorum	Peas Green algae	Oogonia	?	(87)
Venturia inaequalis	*Penicillium* sp.	Perithecia	?	(182)
Phycomyces blakesleeanus	....	Zygospores Zygospores*	Histamine Phloroglucin	(157)
Rhizopus nigricans	Mammals	Zygospores*	Oestrin Theelin	(133)
Melanospora destruens	*Fusarium*, *Botrytis*, etc.	Perithecia	?	(8, 48, 49)
	Lentils	Perithecia	Thiamin Biotin	
Achlya ambisexualis	Lentils	Oogonia	i-Inositol	(136, 138)
Phycomyces blakesleeanus	Potato, oats, malt, agar, etc.	Zygospores	Hypoxanthine (Guanine)	(153, 154)
Zygosaccharomyces sp.	*Aspergillus niger*	Conjugation	Glutaric acid, Riboflavin	(126, 127)

[1]Stimulation of the processes leading to production of the organs indicated, except for the two cases marked by asterisks (*), in which instances the effects were inhibitory.

in the sexual act are contributed by uninucleate free gametes, of which both, one or neither may be motile. Examples: *Chlamydomonas, Ulva, Ectocarpus, Allomyces, Monoblepharis.*

2. Gamete-gametangial copulation, in which one nucleus is furnished by a differentiated uninucleate gamete and the other by a differentiated gametangium which produces no discrete uninucleate gametes. Examples: *Polysiphonia, Achlya, Neurospora.*

3. Gametangial copulation, in which a single pair or many pairs of nuclei are brought together by fusion of differentiated uninucleate or multinucleate gametangia. Examples: *Rhizopus, Pyronema, Ascobolus.*

4. Vegetative fusions initiating stable dicaryons, in which two nuclei originating from undifferentiated vegetative cells retain, once brought together, a dicaryotic association for an indeterminate period prior to fusion. Examples: most of the Basidiomycetes.

The first unequivocal demonstration of sexual hormones in plants was made in 1924 by Burgeff, working with *Mucor mucedo,* a member of the Zygomycetes. Specific sexual substances have been reported, during the subsequent quarter century, in all major groups of algae and fungi except the Rhodophyta and the Basidiomycetes. Since there appears to be no consistent correlation between phylogenetic relationship and the mode of sexual achievement, the order of presentation of the numerous demonstrations of sexual hormones in the algae and fungi here follows the outline of sexual types listed above, with only secondary regard to considerations of phylogeny and chronology.

SEXUAL HORMONES IN GAMETIC COPULATION

All of the algae in which a chemical correlative mechanism of any stage in sexual reproductive processes has been demonstrated belong to the category characterized by fusion of differentiated gametes. These species may be divided, however, into two subcategories, according to the origin of gametes: *a*) species in which the gametes are the immediate products of meiosis, the haploid phase thus being limited to a single cellular generation; *b*) species in which the gametes are the products of a haploid (gametophytic) generation which is capable of extensive or even indefinite asexual reproduction and/or vegetative multiplication. The distinction between these two groups, manifest in totally different life cycles, has in the past been considered to be of fundamental significance from the standpoint of evolution and phylogenetic relationships. The instability under varying environmental conditions of the life cycles of certain algae normally showing a distinct alternation of generations (37, 63, 173) and the demonstration of the efficacy of a single chemical compound in eliminating altogether the gameto-

phyte generation, excepting the gametes themselves, in one algal species (110) would indicate that life cycle patterns may be under rather superficial chemical control and of limited phylogenetic importance.

The first attempt to demonstrate chemical control of a sexual process in any thallophyte sought to elucidate the attraction of spermatozoids to the non-motile eggs of *Fucus,* the gametes of which are the immediate products of meiotic processes. Attraction of the sperm to the ripened egg was suspected of being a chemotactic response to a substance secreted by the eggs. The work of Pfeffer (130), demonstrating the chemotactic attraction of antherozoids to the ripening archeogonium in ferns and duplication of the tactic effect by simple organic acids, particularly malic acid, stimulated the search for an equivalent mechanism in the Fucales.

The early attempts, however, were unsuccessful. Bordet (18) attempted to induce chemotaxis of spermatozoids by a brei of ground eggs, and Robbins (146) tested numerous known chemical compounds for chemotactic activity by introducing the substances in agar in a capillary into a suspension of sperm. In all cases the results were negative.

The lack of chemotactic effect on sperm of the brei of ripened eggs was confirmed in *Fucus serratus* by Kotte (65) who, however, claimed a positive response of sperm to the mucilaginous secretion of the eggs. This effect obtained in cell-free centrifugates of eggs but no attraction of sperm could be elicited by the centrifugate when it was solidified by agar and introduced into the suspension in a capillary. In contradiction to the positive results obtained, the conclusion was reached that the active substance was non-diffusible.

Growth-regulating substances, active in the *Avena* test for auxins, were shown by du Buy and Olsen (34, 128) to be secreted by eggs and sperm as well as by the vegetative thallus of *Fucus,* with the greatest concentration of the substances being secreted by the eggs. This secretion was found to determine the polarity of the developing zygote and greatly to influence subsequent morphogenetic behavior. There was no evidence, however, that this or any other secretion was effective in controlling the sexual process.

Recently Cook, Elvidge and Heilbron (27) have given more definitive information on the attraction of ♂ gametes in *Fucus.*

Ripe eggs were shown to exert a strong attraction to sperm one to two egg diameters away, 0.1–0.2 mm. This effect could be induced through a cellophane membrane. A mucilaginous brei of ♀ fruiting tips induced no chemotactic response in sperm when adjacent drops containing brei and sperm coalesced. When adjacent drops containing sperm and cell-free filtrate of ripe eggs were made to coalesce, a strong positive attraction of the sperm resulted. Contrary to the results obtained earlier by Kotte, introduction of the filtrate of ripe eggs by capillary into a suspension of sperm caused strong attraction, and the effect still occurred when the filtrate was diluted to one fourth of its original concentration. The attracting substance(s) secreted by the eggs was not species specific; a filtrate from eggs of *F. serratus* attracted equally the sperm of the same species, of *F. vesiculosus* and of *F. spiralis*.

The activity of the filtrate of ripe eggs persisted for ten days at 0° C. It was rapidly lost by boiling and by evaporation of the filtrate, even under the conditions of lyophilization. All attempts to concentrate the activity were unsuccessful. The substance is extremely volatile, probably gaseous, and could be boiled out of solution by passing a stream of gas through the filtrate for a few minutes. It could be recovered, however, in a liquid air trap in which a small amount of seawater was frozen.

Further work by Cook and Elvidge (26) sought to isolate and identify the chemotactic substance secreted by the ripened eggs of *F. serratus* and *F. vesiculosus*. In this they have so far been unsuccessful. A number of highly volatile compounds, mostly hydrocarbons and ethers, various constituents of low boiling petroleum ether, diethyl ether, etc., were found to duplicate the activity of the naturally secreted substance. n-Hexane, the most active of these compounds, induced chemotaxis of spermatozoids in a concentration of 10^{-7} to 10^{-6}. Analysis of filtrates from ripe eggs by means of a mass spectrograph indicated a molecular weight of 74 and certain of the structural characteristics of the active substance. The naturally occurring chemotactic substance remains unidentified.

Crystals of indole-3-acetic and naphthalene-acetic acids in sperm suspension rapidly collected clusters of sperm by means of local immobilization (27).

A comparison of enzymatic activities of eggs and sperm of *F. vesiculosus* showed significant and reproducible differences between

♂ and ♀ gametes (Sosa-Boudouil (177)). The importance of these differences in fertilization and subsequent development was suspected but was not explored.

Most of the work on chemical regulation of sexual processes in forms reproducing by gametic copulation, however, has been done on forms in which the gametes are produced by a vegetative, haploid, gametophytic generation. These forms are for the most part members of the Chlorophyta, although significant contributions have in a few cases resulted from investigations on members of the Phaeophyta and Chrysophyta.

The activity of hormones in the copulatory process of planogametes of many algae has been suspected for a long time. A large number of workers, almost all of them German students of the algae, have made many significant contributions to the understanding of sexuality in the algae. This work culminated during the period 1933–51 in the detailed description of the copulatory process in *Chlamydomonas*. Although there is considerable duplication in the observations of the earlier workers, their intensive investigations cover many different forms and indicate in consequence the extent of the regulatory mechanism among all groups of algae reproducing by planogametes.

Jollos, in 1926 (56), demonstrated that the ultimate sexual affinity of the gametes of *Dasycladus clavaeformis* could be affected by the cell-free filtrates of gametes of the same species. When gametes of a weak (−) strain were kept for two hours in the filtrates of a strong (−) strain and then transferred to mating dishes containing (+) gametes, the mating reactions of the (−) gametes were greatly intensified: the gametes now behaved as do gametes of a strong (−) strain. Furthermore, when gametes of a weak (−) strain were similarly treated in the cell-free filtrate of a strong (+) strain, they would no longer fuse with (+) gametes but only with (−) gametes. In both cases the sexual behavior had assumed that of the gametes of the filtrate to which they had been exposed. Treatment in various filtrates of the gametes of strong (+) and strong (−) strains had no apparent effect on their subsequent sexual activities. The author, in seeking to explain these effects, says: "A chemical analysis of the filtrates causing these reversions has not yet been possible and we cannot discuss the action of the filtrates, i.e., whether we deal here with the reaction to a single

specific substance formed by the gametes or with a non-specific change in the culture medium ".

The mechanism of attraction between motile gametes or between a non-motile egg and motile spermatozoids has interested biologists since the time of Bordet's unsuccessful attempt in 1894 to demonstrate its chemical basis in *Fucus* (18). It was only with the work of Geitler, in 1931 (39), on *Tetraspora lubrica,* however, that the many ramifications of the problem became apparent. In this species, as well as in many others described later, clumping of large numbers of gametes, termed atypical group-formation (" atypische Gruppenbildung "), occurs under certain conditions, and it was this behavior of gametes which Geitler attempted to analyse. He demonstrated that group formation in *T. lubrica* depends to a great extent on the quantity of gametes present and also on the proportionality of gametes of the two sexes. A definite minimal number of gametes per unit volume of fluid (a single drop) must be present for group-formation to be initiated; if this minimal concentration is present, groups of 20–30 clumped gametes are formed; if the concentration of gametes is below the minimal, only isolated pairs are formed. Under the proper qualitative and quantitative conditions of mixture, the clumping occurs immediately after mixing of the gametes of the two sexes. A definite attraction of gametes to a group appears to operate through a distance two to three times the length of a gamete, and this attraction is considered by the author to be chemotactic. Perhaps the most significant observation made by Geitler, however, was that the filtrate of a suspension of gametes of one sex introduced into a suspension of gametes of the opposite sex would cause group-formation just as well as the suspension containing the gametes. In groups induced by the filtrate no fusion occurred and the gametes eventually dispersed singly. He further showed that repeated addition of opposite-sexed gametes to a filtrate nullified the group formation potential and that this apparent consumption could be used for quantitative estimation of the group-formation factor in the filtrate.

In the same year Pascher (129) gave a detailed description of the copulatory activities of the gametes of *Chlamydomonas paupera.* The gametes of this species vary considerably in size, but there is apparently no correlation between size and sexual behavior. Following release, the gametes swim around at random for a period,

the duration of which depends upon the type of colony from which they are derived. A single gamete is then seen to become more sluggish than its fellows, and it eventually comes to rest, attaches itself by its flagella to a solid surface and begins to oscillate back and forth. The motile gametes in the immediate vicinity of the lone sedentary gamete then become oriented in respect to it and move toward it. With time the distance through which this attraction is discernible increases until finally the one attached gamete constitutes the center of a group of 100 cells or more. Shortly thereafter some of the previously attracted gametes become sedentary and in turn initiate new group-formation centers. As more of the active gametes become sedentary and assume the role of new centers the groups become progressively smaller so that finally only pairs of gametes remain. The members of each pair then fuse to form a quadriflagellate zygote. In *C. paupera* group formation was assumed to be an expression of a chemotactic reaction in which specific substances, secreted by the sedentary gametes, act upon those still motile, resulting in their orientation and attraction. At subsequent times other gametes, reaching the requisite physiological age, initiate secretion of the substance(s) and become in turn secondary centers of attraction.

A few less detailed observations made by Pascher on *Sphaeroplea* sp. are of equal significance and interest. In this species ♂ and ♀ gametangia are produced on the same thallus, but each filament is restricted to the production of gametangia of only a single sex. Male and ♀ filaments were isolated before maturation of the gametangia and were allowed to mature separately. If then a filament bearing ripe (opened) oogonia was introduced into a suspension of spermatozoids, the ♂ gametes rushed through the pores of the oogonia and swarmed about the eggs. A cotton fiber soaked among ripened oogonia and put into a drop of suspension of spermatozoids caused active attraction and swarming for one to two hours, although the activation was of somewhat less intensity than that elicited by the filament bearing the oogonia themselves. Here the secretion of an attracting substance by the oogonia was quite apparent, and by repeated changes of water over the oogonia it was shown that the secretion of the substance continued for 18 to 20 hours.

Mainx (94), also in 1931, published a description of the copu-

latory behavior of gametes of *Hydrodictyon reticulatum,* in which species he found essentially the same situation as that described above for *C. paupera.* In seeking to explain the observed pattern of behavior he rejected both the more or less equal secretion of an attracting substance by all gametes and the secretion of different attracting substances by each approximate half of the members of the gamete population. He believed, like Pascher, that each gamete has the capacity to secrete the single attracting substance during a period of advanced age and that final sex determination is therefore dependent upon the degree of maturity of a gamete relative to that of its associates. In this connection the similarity of maturation and copulatory behavior of gametes in these algae to the comparable situation in the developmental history of the chytridaceous Phycomycete *Synchytrium fulgens* (84) is pointed out and the assumed hormonal mechanism in the one is used to explain the other. Indeed the two cases are very similar and possibly will eventually be shown to operate by means of similar correlative mechanisms. However, observations on three forms, in which the ultimate sexual reaction apparently depends primarily on the physiological age of the gametes, would seem to be a very narrow base to justify the conclusion, reached by Pascher and Mainx, that the concept of genetic differentiation of male and female sexual cells is outmoded.

Atypical group-formation was reinvestigated in 1934 by Foyn (35, 36) who described the reaction of gametes of *Cladophora subrina* and of *Ulva lactuca.* In both of these species large clumps (groups) of gametes were formed whenever gametes of the two sexes were brought together in sufficient numbers and in the proper numerical ratio.

Hammerling (41), in connection with his work on morphogenesis in *Acetabularia mediterranea* and *A. wettsteinii,* gave a description of gametic copulation in these species. Gametes of either sex alone were observed to move at random, whereas in a mixture of gametes of two sexes group formation occurred, the groups increasing in size until they were visible macroscopically. Conjugation of pairs of gametes followed upon the breaking-up of the large clumps. Hammerling pointed out that certain external factors, such as light and concentration of medium constituents, as well as certain internal factors, such as age, can determine group-formation, mobility and ability to copulate. The effects of the external

factors were reversible, only one to two minutes being required to accommodate to new conditions. Without postulating any specific chemical substances or a well-defined correlative mechanism of sexual regulation, he speaks of "fusion factors", "group-formation factors" and "attraction factors".

Hartmann (45), in describing the sexuality of *Ectocarpus siliculosus,* postulated the activity of two different stimulatory substances from "unripe" and "overripe" gametes respectively. Usually no grouping or copulation occurred when suspensions of ♂ and ♀ gametes were brought together in the late afternoon, particularly if the gametes of one sex were present in small number relative to those of the opposite sex. In a balanced non-copulating mixture of gametes, however, addition of gametes of either sex brought from stock cultures allowed both group-formation and copulation. The stimulatory substances were apparently used up in "overripe" samples of gametes. Hartmann's evidence of two stimulatory substances lies chiefly in the following observations: *a*) isolated ♀ gametes remain motile indefinitely but become sedentary and lose their flagella when ♂ gametes are added; *b*) ♂ gametes, when added to a suspension of ♀ gametes, were strongly attracted to the sedentary ♀'s. Attempts to duplicate these results with cell-free filtrates were, with a single exception, unsuccessful.

The sexuality of various species of *Dunaliella,* a genus of the Chlamydomoneaceae which tolerates extremely high concentration of salt, was the subject of a paper by Lerche (89), who described the general conditions requisite to sexual expression in this unusual genus. Copulation of gametes can occur in darkness, although light is required for the differentiation of gametes. In the copulatory processes in *Dunaliella* certain of the findings of Moewus on *Chlamydomonas* (see below) were confirmed. Two sexual hormones could be demonstrated, although it was recognized that the activity of these hormones was much less intense than in *Chlamydomonas* and that they could be demonstrated only with relative difficulty. Addition of distilled water to well-developed cultures induced the differentiation of gametes. After three days this fluid was concentrated and freed of all gametes by centrifugation. This fluid added to a suspension of gametes of the opposite sexual strain slowly brought about group-formation, beginning in about 30 minutes. Normal green gametes were shown to produce a higher con-

centration of the active substance(s) than red cells, the latter type of cell developing under conditions of nitrogen or phosphorus deficiency. The activity of hormones could be demonstrated only with centrifugates; filtrates passed through a filter membrane were ineffective, even after the addition of bacteria (see below). Addition of active filtrates of *Chlamydomonas eugametos,* obtained from Moewus, to suspensions of gametes showed no effect and was cited as evidence of hormonal specificity.

SEXUAL HORMONES IN CHLAMYDOMONAS. The various investigations on sexuality and hormonal activity in the sexual processes cited above served as a background for a series of investigations which, when and if their results are largely confirmed, would in all probability to be recognized as one of the outstanding developments in the biological sciences of the present century. This work, dealing with the sexuality and the precise mechanism of chemical control of sexual processes in various species of *Chlamydomonas,* particularly in *C. eugametos,* was initiated in 1932 and 1933 by the German worker, Franz Moewus, who later was joined by R. Kuhn, the distinguished carotenoid chemist, and numerous other collaborators. The work has been frequently reviewed in varying detail and degree of scepticism or acceptance by Thimann (178), Murneek (125), Philip and Haldane (131), Sonneborn (176), Schopfer (171), Hartmann (46), Cook (25), Smith (175) and others. Of these, the reviews of Sonneborn, who analyses the work to the year 1940 with critical thoroughness, and of Hartmann, who treats it exhaustively from the bias of its clarification of the near ultimate mechanism of relative sexuality, are particularly recommended. The work is reviewed here in some detail, not only to make the present review complete, but *a*) to point out certain anomalies which have presumably escaped previous criticism, *b*) to include the results of later work, and *c*) to compare the sexual hormonal mechanism of *Chlamydomonas* with corresponding mechanisms in other members of the Thallophytes.

The original papers of Moewus (44, 98) on *C. eugametos* are of particular interest because in these preliminary studies the basic problems of the correlative mechanism in the copulatory process are clearly recognized and appreciated. *C. eugametos* is an isogamous dioecious species, the products of meiosis giving rise by vegetative multiplication to clones belonging to two sexual strains.

Within each clone there is no mating, while in a mixture of cells of compatible clones, copulation occurs freely between individual cells. Vegetative multiplication can be continued indefinitely in the absence of compatible cells and/or the absence of certain physical and nutritional factors which are required for sexual activity. Sexual fusion, however, is the almost immediate result, at any stage in the developmental cycle, of bringing together cells of compatible clones under the specified conditions, i.e., every living cell is a potential gamete and can pass immediately from the vegetative to the sexually active phase upon establishment of the exact requisite conditions.

The primary condition for sexual activity is motility of the cells, and this requires a liquid medium. On a semi-solid medium (agar) the cells remain non-flagellated and of course non-motile indefinitely. Upon transfer to a liquid medium the cells may be induced to produce flagella and become motile by exposing them to light or by providing a sugar in a concentration of about 1% in the dark. Cells in which motility has been induced in the dark, however, are sexually inactive and become capable of copulation only 30 to 40 minutes after being brought into light. Cells in the light are sexually active under those conditions which render them motile.

Further observations concerned secretion into the fluid medium by the sexually active cells of substances which were required for actual copulation. These substances are distinct from the motility substance and are secreted by the cells only in light. Gametes of each of the two sexual strains were shown to secrete a specific substance, and copulation could occur only when both were present. While these specific copulatory substances were secreted only in light, sexually inactive cells kept in the dark could absorb the substances from filtrates of gametes of the same sexual strains which had been illuminated and thus be rendered sexually active and capable of copulation in the dark. This dark absorption of the substance formed by gametes in the light required a minimum of 20 minutes.

Atypical group-formation was also shown to be brought about in each sexual strain by substances secreted into the medium by cells of the opposite strain, but not by those secreted by cells of the same strain. These substances, like the copulatory substances but distinct from them, were secreted only by motile cells in the light, i.e.,

by sexually active cells (gametes), but only after a longer period of illumination than required for secretion of the copulatory substances. Atypical group-formation occurred when gametes of one sex were placed in the cell-free fluid of the other sex, provided *a*) that certain species of bacteria were present and had been present for at least 20 minutes previously, *b*) that the fluid had been cleared of cells by centrifugation. Bacteria alone were invariably ineffective; other species of bacteria and the dead cells of all species tested in the presence of the secretions were likewise ineffective. Passage of the fluid through filters or filter membranes either removed or destroyed the activity of the specific group-formation substances.

Further properties of the group-formation substances were established in this preliminary work and, incidentally, thereafter forgotten or neglected. These substances, unlike the copulatory substances, were not inactivated by light. The activity was destroyed by heating to 50° C for 50 minutes but would withstand temperatures up to 40° C for periods up to 120 minutes, after which activity decreased with time, having disappeared at the end of 15 hours following removal of the gametes from the fluid. A most unusual finding was a marked depression of the freezing point of filtrates containing the active group-formation substances. Active filtrates did not freeze at – 10° C, whereas the filtrates would freeze almost immediately at this temperature after their inactivation. It is surprising that this curious phenomenon was not subsequently investigated in detail.

In the descriptive papers published prior to 1940, Moewus (98, 99, 102, 103, 106) reported a number of other findings which have significance in the description of the hormonal mechanism which follows. A number of species of *Chlamydomonas* which would freely interbreed with *C. eugametos* were described and the pattern of sexual segregation and inheritance determined in the various interspecific matings. This group of interbreeding species, comprising *C. eugametos, C. dresdensis, C. braunii* and *C. coccifera,* include isogamous, anisogamous and heterogamous forms, and it was thus possible throughout the interbreeding group to make definite ♂ and ♀ assignments to sexual strains, even where there was no morphological, only physiological, differentiation between the sexes.

Among this group of interbreeding species there were also marked variations in sexual behavior, sexual segregation and sexual determination. In dioecious species the two sexes are strictly segregated at meiosis and the products of the meiotic process give rise to two types of clones, ♂ and ♀, in equal frequency, each clone being self-sterile. In monoecious species both sexes are regularly present in every clone in approximately equal numbers, each clone thus being self-fertile. It usually happens in monoecious forms, however, that after completion of copulatory activity within the clone, a varying number of residual cells are left unfused, and these for any culture are always of the same sex, but for a number of cultures of the same clone the residual cells are ♂ and ♀ in about equal incidence. Finally, in subdioecious (originally designated subheteroecious by Moewus) species the condition is intermediate between the two preceding types; each culture contains both ♂ and ♀ cells, but for a given clone cells of one sex are always present in much greater number than those of the opposite sex. Subdioecious clones may be designated as either ♂ or ♀, depending on the predominant sex.

In dioecious forms sexual determination is genetic and is termed "genotypic", i.e., the sexual affinity of any cell is determined genetically at meiosis. In monoecious and subdioecious forms sexual determination may be partially or completely determined by factors in the environment and is termed "phenotypic".

A further effect of filtrates on the sexual process, originally discovered by Jollos (56), was confirmed by Moewus and collaborators. Filtrates from sexually active gametes were shown to be capable of determining completely the mating affinities of the cells of monoecious clones and to a lesser degree those of the cells of subdioecious clones. Filtrates from a suspension of ♂ gametes caused all cells of a monoecious clone to react only as ♂'s; filtrate of ♀ gametes acted similarly, making all cells react only as ♀'s. Filtrate of a suspension of gametes of a single sex had a partial effect on a subdioecious clone: if the predominant sex of the clone was the same as that of the gametes from which the filtrate was obtained, all cells would react alike and as the gametes which produced the filtrate; if the predominant sex of the clone was opposite to that of the gametes from which the filtrate was obtained, only about half of the cells would be transformed, and the subdioecious

clone would have the characteristics commonly exhibited by a monoecious clone. These substances were secreted by all motile cells under the conditions required for motility induction and were called "termones".

Within the complex of species interbreeding with *C. eugametos* it was clearly evident that all clones of a single sex were not equally potent in their sexual reactions. Interbreeding experiments showed that each sex consisted of a number of distinct sexual strengths or valences. At first four valences of each sex were recognized, but later in the work exceptionally strong mutant ♂ and ♀ clones were described, thus totalling ten different sexual strains (valences). These range strong ♂ → weak ♂ → weak ♀ → strong ♀ and are designated *♂ 5*, *♂ 4*, *♂ 3*, *♂ 2*, *♂ 1*, *♀ 1*, *♀ 2*, *♀ 3*, *♀ 4* and *♀ 5*. Mating reaction occurred not only between ♂ and ♀ strains but also between certain valences of the same sex. When the strains of the different valences are arranged in serial order as above, mating will occur in all of those combinations in which the strains differ by two or more plus the combination *♂ 1* × *♀ 1*, which is also fertile. The intensity of the mating reactions, particularly group formation, increases as the difference of valence numbers between the two contrasted strains is increased.

The findings set forth above constitute the ground work for a detailed description of the hormonal mechanism correlating sexual processes in *Chlamydomonas* as communicated in numerous papers appearing from 1938 to the present. It is impractical to consider here the tremendous amount of material contained in these papers, either in comprehensive detail or in chronological order. Instead, the hormonal mechanism as currently interpreted by the authors (69, 118, 119) will be presented, followed by a consideration of numerous findings which either *a*) contradict or at best find no satisfactory explanation in the current interpretation, or *b*) are subject to critical question on the basis of biological, chemical or physical phenomena in general.

THE HORMONAL MECHANISM IN CHLAMYDOMONAS. The several distinct and sequential stages through which cells of *Chlamydomonas* progress in passing from the vegetative condition to zygote formation are summarized in Fig. 1. The specific chemical compounds which regulate the sexual processes are the successive products of the enzymatic degradation and/or synthetic reactions comprising two complex biochemical systems, one involving caroti-

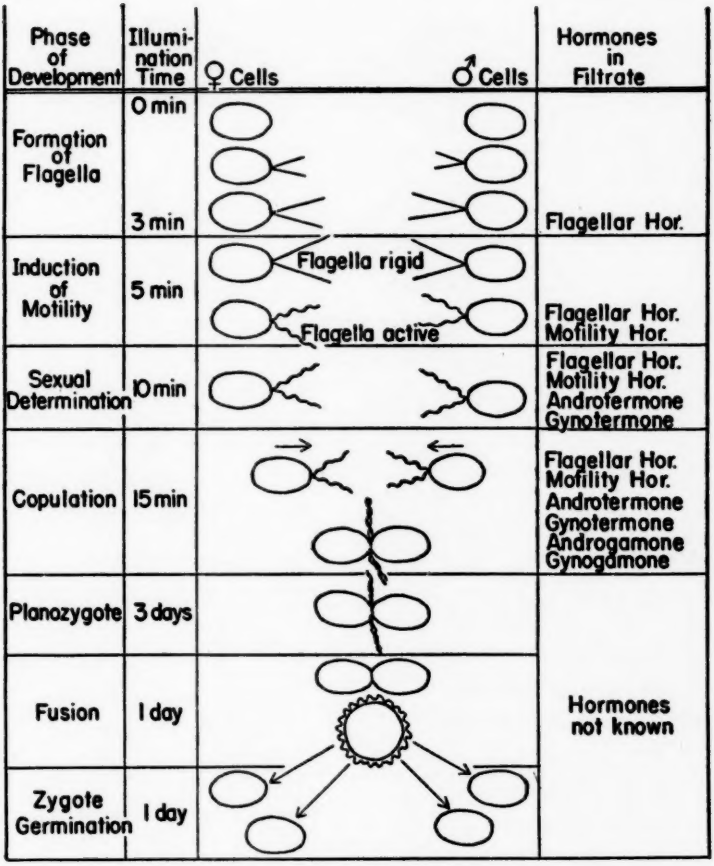


FIG. 1. Sequence of stages in the sexual interaction of male and female cells of *Chlamydomonas eugametos* f. *phlegmatikos* (adapted from Moewus, 1951).

noids, the other flavonols. Three schemes, recently published by Moewus (119) to account for the origin of the various substances which initiate and control the formation of flagella, motility, sexual determination, gametic copulation and inhibition of gametic copulation, are combined in Fig. 2. The specific biochemical reactions known to be controlled by single genes are indicated by the original symbols for the responsible genes.

Figures 1 and 2 summarize the principle aspects of the knowl-

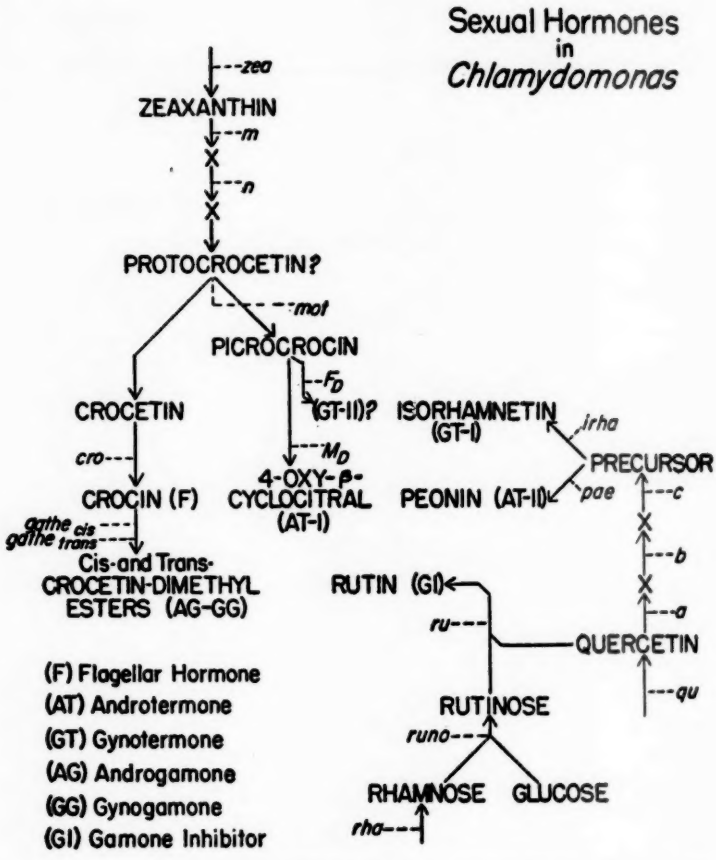


FIG. 2. Scheme of the biosynthetic reactions leading to production of the various hormones involved in the sexual reactions of *Chlamydomonas* (adapted from Moewus, 1951).

edge and current interpretation of sexually active substances in *Chlamydomonas* and are included here as a means of orientation for the following outline, since the description of certain stages requires reference to other stages yet untreated.

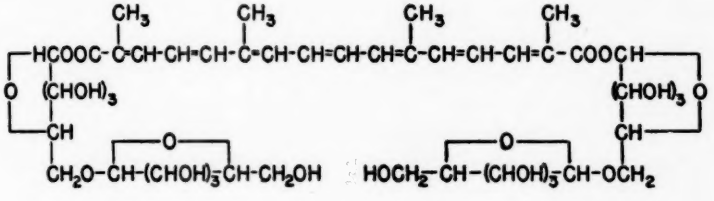
1. The Flagellar Hormone: Exposure of vegetative cells in water to light of any wave length of the visible portion of the spectrum results in the formation of flagella and, shortly thereafter, in initia-

tion of motility. In the dark these effects can be duplicated under aerobic conditions by 1% sugar and under anaerobic conditions by the filtrate of motile cells. The effect of such filtrate is quite spectacular: the flagella are fully formed within three minutes, and at the end of five minutes the cells are capable of motility (105).

The processes of flagellar formation and motility, however, are distinct. A short period elapses after the flagella are fully formed, during which time the flagella are rigid, before the cell becomes motile. That the effect upon non-flagellated cells in the dark of the filtrate of either flagellated cells not yet motile or flagellated cells which had assumed motility corresponded exactly with the condition of the cells from which the filtrate was taken proved the two phenomena to be distinct and to depend upon different chemical agents (113). All cells in water under the conditions of illumination, etc. which induce motility secrete the flagellar-inducing substance. This hormone appears to be neither race- nor sex-specific.

A number of single gene mutant clones, which exhibit abnormalities involving the flagellar hormone, have been recognized and used in the characterization of the hormone. Two mutant clones, nos. 5 and 6, produce the flagellar hormone in abnormally high quantities while secreting neither gamones nor termones. Two other categories of genic mutants, *cro*^{0} and *mot*^{0}, secrete no flagellar hormone and remain non-flagellated in light. These abnormal manifestations result from genic blocks of the carotinoid biochemical chain subsequent and prior, respectively, to the stage of the active substance itself.

The flagellar hormone has been approximately identified as crocin, the digentiobiose glycoside of crocetin, a 20-carbon carotinoid:



CROCIN

Although crocin per se has not been isolated from *Chlamydomonas* cells, the evidence that it is the active substance is extensive, though not positively conclusive, viz.:

a) The concentrated filtrate of 317 liters of culture of motile cells contained a pigment which could not be spectroscopically distinguished from crocin isolated from safran (the dried pistils of flowers of *Crocus*) (80, 105).

b) The conditions of hydrolysis and the chemical and spectroscopic characterization of the hydrolytic products of the hormones agreed in detail with those of crocin.

c) The biological effect of the filtrate was exactly duplicated by crocin.

d) From 8.5 gm. of dried cells of a race producing an abnormally high quantity of the active flagellar substance, mutant no. 5 above, a water-soluble orange-red pigment was extracted which, following hydrolysis and methylation, yielded 40 mg. of trans-crocetin-dimethyl-ester (74).

The *cro*^{0} mutant was used to determine the effective concentration range of crocin. This mutant, deficient in flagellar hormone synthesis, produces flagella only when furnished with an external source of the active substance. In a suspension of cells containing 2×10^6 cells/ml. (the standardized suspension density used throughout the work), a concentration of crocin of 4×10^{-11} was required to bring about production of two flagella on all cells. Thus approximately 10^4 molecules per cell are required for complete activation. Earlier reports of flagellar production by 1.25 molecules/cell (105, 108) have since been shown to apply only to normal cells having pyrenoids. These organelles possibly serve to store a preformed reserve of the active substance, or they may constitute an essential part of a mechanism for the rapid synthesis of the substance following stimulation by crocin in exceedingly low concentration. Whatever the mechanism may be, the titer of crocin increases a thousandfold within a few minutes after addition of the " primer ". The production of the flagellar factor by cells of *C. eugametos* lies between ten and a hundred times the amount required for their own activation.

Identity of crocin as the flagellar hormone apparently obtains for *C. eugametos* but not for the other species investigated. In other

species the reaction to crocin required a longer period or a higher concentration or both. The preferential effects of different di- and trisaccharides, cellobiose, gentiobiose, cellotriose, etc., in inducing formation of flagella in the dark in different species indicate that in each species a species-specific glycoside of crocetin is naturally produced (80, 105, 107).

Production of crocin is thought to result from a coupling of crocetin with two molecules of gentiobiose by an enzyme controlled by the gene *cro*. Crocetin, in turn, is postulated as one of two hydrolytic products of the enzymatic cleavage of protocrocin, a reaction controlled by the gene *mot* (78).

The chemical specificity of crocin is quite marked; its activity has not been duplicated by any one of many other compounds tested, including crocetin, the dimethyl esters of crocetin, and other carotinoids.

2. The Motility Hormone: Relatively little is known of the motility hormone. It is secreted by all cells within two or three minutes after their production of flagella. Its specific activity is to render the newly formed flagella active, thereby inducing motility. The active substance, in contrast to crocin, is light-stable. No mutants were found by Moewus and associates, in which its secretion or specific activity are affected. Lewin (90*a*), however, has reported mutant clones, in which the motility mechanism is impaired. In these mutants flagella are formed but they remain rigid.

Induction of motility in newly flagellated cells was originally considered to be a phase in the same response which induces formation of flagella, thus initiated also by crocin (80). Subsequent studies on crocin activity, however, showed that certain samples of crocin (isolated from safran) induced motility as well as flagellar formation, whereas other samples did not. It was therefore assumed that a contaminating substance in the active crocin sample was responsible for the specific activity (113).

Nothing is known of the chemical nature of the motility factor beyond the fact that it can not be replaced by any of the known members of the carotinoid series (119).

3. The Sex-Determining Hormones, The Termones: Filtrates of cultures of motile cells of dioecious species and of either sex contain hormones which are capable of determining completely the sexual behavior of monoecious cells. Thus monoecious cells placed

in the filtrate of ♂ dioecious cells will thereafter react only as ♂ gametes; filtrate from ♀ dioecious cells have the corresponding ability to determine monoecious cells as ♀. The effects of such filtrates on subdioecious clones, as described above, is somewhat more complicated and depends upon the predominant sex of the subdioecious clone as well as the sexual type of the dioecious clone from which the filtrate was taken. The effect of these agents is temporary, the induced sexual determination persisting only as long as the active substances are present in the medium (119).

These sex-determining effects upon cells in which sexual type is not determined genetically have been shown to be the specific activities of three known compounds, and probably of a fourth one which is still unidentified. Two of the compounds, secreted only by ♂ cells and causing the determination of monoecious cells as ♂, have been designated "androtermones"; the corresponding compounds, secreted by ♀ cells, were designated "gynotermones". Two of the termones, androtermone II and gynotermone I, are alternate, common-precursor products of the flavonol sequence, while the other hormones, androtermone I and gynotermone II (?), are probably alternate common-precursor degradation products of the carotinoid chain.

Interpretation and understanding of the mode of origin and chemical nature of the termones of the carotinoid series have undergone a number of revisions since the system was first described. In the original interpretation protocrocin, a hypothetical precursor of crocin, was considered to be hydrolyzed enzymatically, under the control of gene *mot*, to two molecules of picrocrocin and one molecule of crocin. Picrocrocin, the glucoside of safranal, showed definite activity in determining monoecious cells as ♀ and was considered to be closely related to the naturally secreted gynotermone. Picrocrocin, however, had only about one thousandth the activity of the secreted hormone and was not claimed to be the actual gynotermone; the naturally occurring factor was suspected of being another glycoside of safranal containing a sugar other than glucose.

Enzymatic hydrolysis of picrocrocin could be accomplished by ♂ cells, though not by ♀, to yield glucose and safranal. Safranal, when added to monoecious cells, would duplicate the activity of the naturally secreted androtermone and was considered to be identical to that hormone. The activity of safranal was quite specific; a

number of closely related compounds, including citral, α-cyclocitral and β-cyclocitral, showed no androtermone activity (109). It was later shown that 4-oxy-β-cyclocitral (4-oxy-2,6,6-trimethyl-Δ′-Tetrahydrobenzaldehyde) was even more active than safranal (70). 4-oxy-β-cyclocitral, the hydration product of safranal, is now considered as one of the two naturally occurring ♂-determining secretions and has been designated "androtermone I". About 50,000 molecules of androtermone I per cell are required to demonstrate complete determination of monoecious cells, whereas about ten times as much safranal is required for equal activity (114).

Hydrolytic cleavage of picrocrocin is due to the activity of an enzyme controlled by gene M_D, located on the 10th chromosome and closely linked to the male sex factor, M. Some 43 specific mutations at the M_D locus were induced, and in these mutated forms the functions associated with M_D were lost without interfering with other functions or characteristics of the affected cells (78).

The gynotermone activity of picrocrocin is also differently interpreted as the result of more recent work. There is good evidence, chiefly from reactions involving two mutants, $F_D{}^0$ and *irha*^{0}, both of which affected loci are closely linked to the female sex factor, F, that picrocrocin is enzymatically transformed (F_D) into a compound having gynotermone activity. This probable hormone has tentatively been designated as "gynotermone II", although its occurrence and activity have not as yet been rigorously proved (119).

Paralleling the sequence of biochemical reactions which produce androtermone I and gynotermone II is a completely distinct series of reactions which produce two other substances having equal sex-determining activities. In this second biochemical chain a number of reactions, beginning with production of quercitin, the first known intermediary, eventually yields one or the other of two active compounds, isorhamnetin, a flavonol, or peonin, an anthocyanin. These two compounds apparently have a common precursor which in ♀ cells is converted by an enzyme controlled by the gene *irha* to isorhamnetin, "gynotermone I", and in ♂ cells by an enzyme controlled by *pae* to peonin, "androtermone II". The loci *irha* and *pae* are closely linked to the two sex factors F and M, respectively, which genetically determine sexual affinity in dioecious species.

Isorhamnetin has been confirmed in the following manner as the

actual gynotermone: *a*) In 1942 (76, 77) there was isolated from the pollen of crocus a flavonol glycoside which had the peculiar property of causing flagellated cells of *Chlamydomonas* to lose their flagella. When the glycoside was hydrolyzed, however, the aglycone flavonol no longer had this activity, but it was found that it was exceedingly active in determining monoecious cells as ♀. This flavonol was isolated and identified as quercetin-3-dimethyl-ether or isorhamnetin (71). This crystalline compound, when furnished to monoecious cells at the concentration level of 50,000 molecules per cell, showed complete sex determination activity (114). Isorhamnetin was thus exceedingly more active than picrocrocin. It also showed a high degree of specificity in the reaction, for of 45 flavonols and flavonol derivatives tested, only isorhamnetin showed the characteristic activity (81). *b*) From five gm. of dried cells of a mutant clone (no. 3) which showed exceptionally high gynotermone activity, 70 mg. of crystalline isorhamnetin were isolated (73, 74).

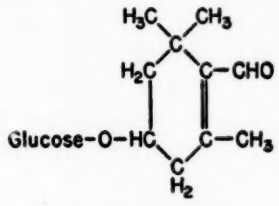
Peonin was similarly confirmed as the actual androtermone: *a*) An anthocyanin pigment, isolated from the berries of buckthorn and identified as peonin, was shown to determine monoecious cells as ♂. Peonin alone of a number of tested anthocyanin pigments, including cyanidin, violanin, malvin and cyanin, exhibited this activity (12, 13). *b*) From 17 gm. of dried *Chlamydomonas* cells of an induced mutant, which produced none of the hormones derived from the carotinoid chain (flagellar hormone, gamones or androtermone I), peonin was isolated in the amount of 53 mg. (75). Peonin, when tested with monoecious cells, was shown to have androtermone activity almost exactly equivalent to that earlier shown for 4-oxy-β-cyclocitral, requiring about 50,000 molecules/cell for complete sex determination.

The common precursor of isorhamnetin and peonin is thought to be a diglycoside of isorhamnetin which in the presence of the *irha*-controlled enzyme (♀) is hydrolysed to isorhamnetin, while in the presence of the *pae* enzyme (♂) is reduced to peonin. These two enzymes exhibit maximal activity at different hydrogen ion concentrations, *irha* in acid medium and *pae* in alkaline medium, respectively. This difference in pH optima of the two enzymes would account, according to Moewus (118), for the sex-determining effects of acid and alkaline conditions in certain mo-

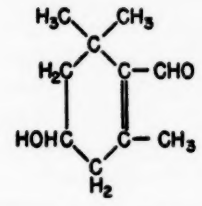
noecious races, e.g., *C. eugametos* f. *alpina*. In this race, cells grown or suspended in a medium at pH 9.5 produce only ♂ gametes, while at pH 4.5 only ♀ gametes are produced.

One additional phenomenon observed in monoecious clones is explicable in terms of the postulated chemical characteristics of the termones. Boric acid in low concentration, M 10^{-10}, causes all cells of monoecious clones to be differentiated as ♂ gametes. Isorhamnetin has been shown to form an inactive complex with boric acid which effectively removes the gynotermone from the physiological system, leaving the cells subjected only to the determining effects of peonin (and 4-oxy-β-cyclocitral?). Thus boric acid has the effect of an inorganic androtermone (77).

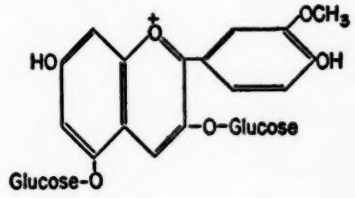
The comparative activities of the termones of the two series, carotinoid and flavonol, are still imperfectly understood, and the entire problem of sex-determining substances is the subject of continuing experimentation. The chemical structures of the various termones would indicate their participation in dissimilar biochemical processes:



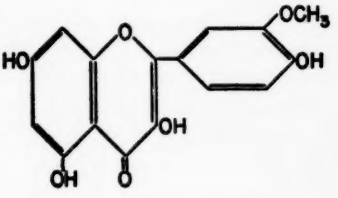
Picrocrocin



4-Oxy-β-cyclocitral



Peonin



Isorhamnetin

Whether there are in fact alternate independent or parallel interdependent systems involving the two dissimilar classes of termones

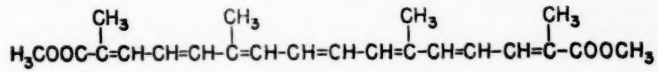
has not been established. That interdependent processes are involved is indicated by Moewus in a recent review (118), in which are cited the broad results of experimentation, details of which have not yet been published. Mutant monoecious clones were found which were incapable of synthesizing either androtermone I or androtermone II; all cells of such clones were determined as ♀ gametes. When 4-oxy-β-cyclocitral was furnished to the mutants no longer capable of producing androtermone I or when peonin was furnished to mutants no longer capable of producing androtermone II, gametes were differentiated as ♂. These results indicate that both androtermone I and androtermone II are required for ♂ determination in monoecious cells, but the specific activities of the two distinct termones could not be distinguished.

4. The Copulatory Hormones, The Gamones: The final hormones which have been demonstrated in the normal sexual mechanism of *Chlamydomonas* are those required for actual copulation of gametes, the gamones. They are produced by ♂ and ♀ cells of all valences under the same conditions inducing motility in light with the single exception that for gamone production blue or purple light is required (vs. any wave length of visible light for the production of flagellar and motility hormones). The filtrate of cells capable of copulation will induce cells maintained in the dark to copulate, but only if the cells and the filtrate are of the same sex and valence. Thus, since there are eight or ten different sexual valences, it would appear that there are many different gamones characteristic of the different strains. Actually only two compounds, occurring in different proportions, have been shown to be present in all cases.

It was demonstrated early in the biochemical work with *Chlamydomonas* that blue or violet light (4961 and 4358 Å) was required for production of the copulatory substances but that they were rapidly inactivated by continued exposure to light of these wave lengths (78–80, 105–107). It was also shown that a filtrate of ♀ cells of valence 4 would, following increasing periods of illumination, successively render cells of ♀ 3, ♀ 2, ♀ 1, ♂ 1, ♂ 2, ♂ 3 and ♂ 4 capable of copulation, after which the filtrate was inactive. These findings indicated the presence of only two substances, conversion from one to the other being accomplished through a photochemical reaction requiring blue or violet light. Cells of both sexes

and all valences can produce in the dark or in red light a precursor which, on exposure to blue or purple light, is transformed into a stable end product at a rate which is a linear function of time. Careful adjustment of the lamp–solution distance permitted a conversion rate of 1%/minute, and this light intensity has been used as the standard throughout all subsequent work (105).

The precursor and stable end product were shown to be two isomeric forms of crocetin-dimethyl-ester, respectively. The dimethyl ester of crocetin is thought to be produced by hydrolysis and re-esterification of crocin, the flagellar hormone. Hydrolysis and esterification of crocin depend upon one or more enzymatic reactions controlled by two series of alleles designated *gathe*$_{cis}$ and *gathe*$_{trans}$. The dimethyl ester of crocetin has the formula:



DIMETHYL ESTER OF CROCETIN

The specific activity of the filtrate of each sexual strain, or valence, was shown to depend upon the ratio of precursor/end product, i.e., the ratio of cis/trans crocetin-dimethyl-ester, as shown in Table II. The proportionality of cis to trans isomers which is able to activate the cells of each valence has the very narrow limits of about ± 1%. Continuous transformation of cis to trans isomer under continuing illumination with blue or purple light thus changes the ratio with time, and at successive and critically specified times the ratio corresponds briefly with that required for sexual activation of each of the serially ordered valences. In the series ♀ 4 → ♂ 4, irradiation periods of ten minutes were required to change the specific activity of the filtrate from that of one valence to that of the valence next in the series to the right with the exception of the transformation from ♀ 1 to ♂ 1 which required 30 minutes. From these results precursor/end product ratios were assigned to the several valences, as shown in Table II.

Evidence for the identity of the cis-isomer as the precursor, the trans-isomer as the stable end product, and the various mixtures of cis and trans isomers as the naturally produced gamones consisted of the following findings: *a*) The cis isomer of crocetin-

TABLE II

SPECIFIC RATIOS OF CIS/TRANS CROCETIN-DIMETHYL-ESTER AS THE GAMONES OF *Chlamydomonas*

(Data, exclusive of last line, from Moewus [119, etc.])

Sex:	Female				Male				
Valence:	4	3	2	1	1	2	3	4	
Interval of irradiation in minutes	10	10	10	30	10	10	10	5	
Precursor/end product ratio	95/5	85/15	75/25	65/35	35/65	25/75	15/85	5/95	0/100
Cis/trans ratios duplicating gamone activity in line above	95/5	85/15	75/25	65/35	35/65	25/75	15/85	5/95	0/100
Rate of cis-trans conversion in % remaining cis isomer per minute	1.16	1.31	1.5	2.78	3.84	6.25	20.0	100	

dimethyl-ester is known to be photochemically transformed to the trans isomer by light of the same wave lengths which transform the precursor to the stable end product (83). *b*) The color, solubility and characteristic absorption bands (4350 and 4630 Å) of trans crocetin-dimethyl-ester agree with those of the stable end product as determined on a concentrated sample of filtrate containing the end product (80). *c*) The effects of various mixtures of cis- and trans-crocetin-dimethyl-ester exactly duplicated the effects of precursor/end product mixtures obtained by irradiation of a filtrate containing the precursor (106). These lines of evidence would appear, in the absence of isolation and identification of both precursor and end product from the proper filtrates, to constitute the best possible evidence of the presence and activity of the cis and trans forms of crocetin-dimethyl-ester as the naturally occurring active gamones.

These substances are claimed to be active in low concentration. Crystalline cis and trans isomers, in appropriate mixtures, exhibited gamone activity in a concentration of 3×10^{-11} (ca. 10^{-11} molar or 2.5×10^4 molecules per cell) (105). This is the concentration of the sum of the two isomers; each, therefore, exhibits its activity at values approaching one hundredth this concentration when the cis/trans ratio is very high (♀ 5, 98.2/1.8) or very low (♂ 5, 1.8/98.2).

Each race and each sexual strain secretes cis- and trans-crocetin-dimethyl-ester in the specifically required ratio for its own sexual union. Thus the two sexual strains of *C. eugametos* f. *simplex* secrete the cis/trans esters in ratios of 1 : 3 and 3 : 1, these strains being ♂ of valence 2 (25/75) and ♀ of valence 2 (75/25), respectively. The sexual strains of other dioecious races, characterized by other valences or sexual strengths, require quite different specific mixtures: 2 : 1 *vs.* 1 : 2, 6 : 1 *vs.* 1 : 6, 18 : 1 *vs.* 1 : 18 (106).

The ratio of the two esters secreted by each race and sexual strain is genetically determined (79). The ultimate determination of cis/trans ratio is fixed by the particular primary sexual factor (realizer), of the multiple allelic series M^4, M^3, M^2, M^1, F^1, F^2, F^3, F^4 characteristic for the particular clone. The mode of interaction between these primary sexual factors and the genes, $gathe_{cis}$ and $gathe_{trans}$, which control the final enzymatic hydrolysis and esterification of crocin to produce the two isomeric esters (78), is not clear.

The cells of two dioecious clones, of the same race or of different races, will copulate when brought together if their characteristic cis/trans ratios differ by 20/20 or more, e.g., 95/5 *vs.* 75/25 (108). Group formation, the agglutination of gametes prior to formation of individual pairs, also depends upon cis/trans ratio differences. When suspensions of gametes differing by 20/20 are mixed, only single copulating pairs result; if the differential is 30/30, groups of 20–30 cells are formed; if the difference is 60/60 or more, large groups of 100 or more cells are formed prior to their ultimate dissolution into copulating pairs.

The gamones show the further activity of inducing chemotaxis of gametes (108). This effect is in certain measure likewise exhibited by motile cells of each species, regardless of their current sexual status, by the specific sugars most active in inducing their motility. Thus gentiobiose, cellobiose and cellotriose in concentration of M 10^{-4}, applied in a capillary at one side of a drop of cell suspension, chemotactically affect cells of *C. eugametos, C. dresdensis* and *C. braunii,* respectively. Motile cells of all species, however, show various responses to the gamone constituents, depending upon their stage of development. Crocin dark cells (motile nonsexual cells) react positively to cis- and trans-crocetin-dimethyl-ester mixtures regardles of the ratio, including cis/trans ratios of

100/0 and 0/100. Young gametes do not show chemotaxis to cis/trans mixtures having ratios greater than 80/20, but react to all mixtures lying between this value and 0/100. Sexually reactive gametes, suspended in a solution containing for each valence a definite and characteristic proportionality of cis and trans isomers, react to all mixtures of which the ratios differ by 19/19 or more from their own characteristic ratio. The intensity of the reaction and the speed with which chemotaxis occurs are proportionate to the difference in ratio values.

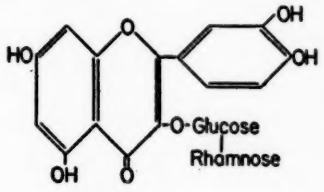
The relationship between chemotaxis and pre-copulation group formation and atypical group-formation, i.e., the responses elicited by the cis/trans ester mixture, on the one hand, and the specific bacteria-requiring group-forming substances, on the other, has not been clarified. In earlier papers much emphasis was placed on the distinction between the atypical group-forming substances and the gamones, as regards both origin and specific activities, but no further information has appeared in later publications.

5. The Copulation-Inhibiting Hormone: A variety of *C. eugametos,* corresponding with f. *typica* in morphology but showing no gametic copulations, was isolated from soil about 15 years ago; this non-copulating variety had been designated f. *agametos* (115). Female and ♂ cells of *typica* were shown to lose their ability to copulate when put into filtrates of cells of *agametos,* indicating the secretion and activity of a copulation-inhibiting hormone, the effect of which was not sex-specific. Formation of flagella and assumption of the motile state were not affected. The cells of *agametos,* however, were capable of copulating for a few minutes when they were first illuminated, following three days of darkness, or when suspended in a saturated solution of cis-crocetin-dimethyl-ester. Under these experimental conditions, cells of ♂ and ♀ clones of *agametos* could be cross-mated with the appropriate clones of *typica.* Genetic analysis of the resulting hybrid zygotes showed the production of the inhibiting substance to be controlled by a single gene, located on the fourth chromosome and subsequently designated by the symbol *ru* (116, 117).

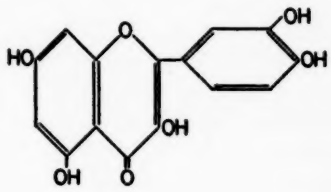
It had earlier been shown, in tests to determine the effectiveness of 45 different flavonols and flavonol derivatives as gynotermones, that rutin (quercitin-3-rutinoside) had strong activity in inhibiting copulation in *Chlamydomonas* (81).

Rutin has been isolated in crystalline form in the quantity of 290 mg. from 4.2 gm. of dried cells of a mutant (no. 1) of f. *agametos* which elaborated abnormally large quantities of the inhibiting substance but which produced neither flagellar hormones, termones nor gamones. This preparation of rutin was identical with other samples of rutin isolated from buckwheat leaves, seed of *Rhamnus utilis*, etc., both in chemical properties and in biological activity (72, 74). Rutin demonstrated complete inhibition of copulation in a concentration of 10^{-10} or 50,000 molecules per cell (115).

The formulae for rutin and its precursor, quercetin, are as follows:



Rutin



Quercetin

The probable biochemical origin of rutin is indicated by a study of cells of a mutant clone (no. 2) derived from the mutant used for isolation of rutin. This mutant involved the genic change $ru^+ \rightarrow ru^0$ and could no longer produce rutin. From five grams of dried cells of this clone, 100 mg. of quercetin were isolated, and this substance was considered as the immediate precursor of rutin, the enzyme controlled by *ru* affecting the coupling of quercetin with rutinose (74). The genic control of the synthesis of rutinose has also been determined and is indicated in Fig. 2. The specific activity of rutin as a copulation-inhibitor can be quantitatively destroyed by boric acid through the formation of an inactive complex similar to that formed with isorhamnetin, gynotermone I (119).

The copulation-inhibiting activity of rutin is operative through its effect upon the production of cis-crocetin-dimethyl-ester, possibly by poisoning the enzyme involved in the final esterification of the gamone (118).

The works and claims of Moewus, Kuhn *et al.* have been subjected to frequent scrutiny and in a number of cases to severe criticism. Most of these criticisms have dealt with various aspects of

the genetic work which parallels and underlies the biochemical and physiological studies immediately concerned with the hormonal mechanism. This portion of the work lies outside the scope of the present review; the reviewer feels, however, that there are a number of details dealing with the hormonal mechanism per se which demand clarification, interpretative revision or experimental confirmation before the work can be accepted in its entirety. The more obvious and important of these uncertain areas justify a brief discussion.

The gamones, cis- and trans-crocetin-dimethyl-ester, the mode of their origin and their biological activities constitute, as originally and currently interpreted, perhaps the most remarkable system known to present day biological sciences. An examination of certain aspects of the system, however, leads to near-inescapable conclusions which would seem to render the entire system unworkable.

Foremost among these uncertainties, first pointed out by Thimann (178), is the foundation upon which the cis/trans edifice is erected: the linear transformation with time of precursor to end product and the assumed corresponding behavior of cis-crocetin-dimethyl-ester under the same conditions. From the oft-repeated tabulation of irradiation time vs. precursor/end product ratio (determined by gamone activity and exactly replacable by cis and trans isomers in definitive and critical proportionalities), the quantity of unconverted precursor at any time during irradiation is given by the formula

$$X_t = X_0 - t,$$

where X_t is the percentage of precursor (i.e., cis isomer) at any time, t, in minutes following the initiation of irradiation and X_0, the percentage of precursor initially present. This is a zero order reaction and could obtain only under certain necessary conditions. These conditions are activity of a catalyst and excess of the reacting compound at all times. Even under these conditions, at some point in the total conversion of cis to trans isomer, the concentration of the cis must of necessity decrease to a limiting value, and at such time the pattern of conversion must depart from the linearity which previously obtained. The presence of a substance(s) capable of catalyzing the cis → trans conversion in the untreated precursor-containing filtrate from motile non-sexual cells appears

not too unlikely, but it does appear exceedingly unlikely that at all times in the conversion the cis isomer should be in excess of such a catalyst. As the cis/trans ratio approaches the values required for ♂ 4 and ♂ 5 activity, the molar concentration of the cis isomer approaches a value of 10^{-13}, and, since no departure from linear conversion is seen up to this time, a fantastically low concentration of the catalyst must be assumed.

The photochemical conversion of cis → trans could more reasonably be expected to show an exponential pattern with time under constant illumination. Data presented by Hausser and Kuhn (47), relative to the quantum efficiency of the photochemical transformation of cis-crocetin-dimethyl-ester to the trans isomer, largely confirms this expectation. The pertinent data of Hausser and Kuhn are of considerable interest, since the conversion of cis to trans isomer assumes such importance in the hormonal mechanism and because the claim of linear transformation is contradicted by the pattern of conversion of the cis isomer found in connection with the quantum efficiency study. These data are included here:

$N_{cis} \times 10^{-16}$	Wave length A	Absorbed quanta $\times 10^{-16}$	Percent transformed	Quantum efficiency
2.28	4350	53.7	17.5	123
"	"	102.1	30.6	126
"	"	196.1	48.2	144
"	"	377.1	63.4	235
2.28	3660	30.1	10.0	124
"	"	59.9	17.2	150
"	"	120.5	31.4	127
"	"	251.0	52.0	131

A plot of the log cis isomer remaining unchanged (ordinates) against increasing value of quanta absorbed (abscissae) yields a very acceptable experimental exponential curve (Fig. 3). A comparable plot of the pattern required for linear conversion, equated with the exponential curve at 10% conversion, 0 – 10% conversion covering the range of minimal divergence between the two functions, illustrates the essential discrepancy.

Consideration of the alleged linear pattern of conversion of cis to trans isomer as regards another aspect yields an interesting and inescapable conclusion. If the percentage of unchanged cis isomer is, as is claimed, an inverse linear function of time of irradiation, it follows that the rate of conversion, i.e., the per cent conversion

of cis per unit time, becomes strongly time dependent. Thus, of the remaining unchanged cis isomer, 1% per minute is converted at the beginning of irradiation, at 75 minutes after initiation of irradiation this rate has increased to 6%, while a rate of 100% per minute is required at the time of final exhaustion of the cis isomer. The conversion rates, expressed as per cent/minute, of the cis isomer at the times and specific ratios corresponding to the successive

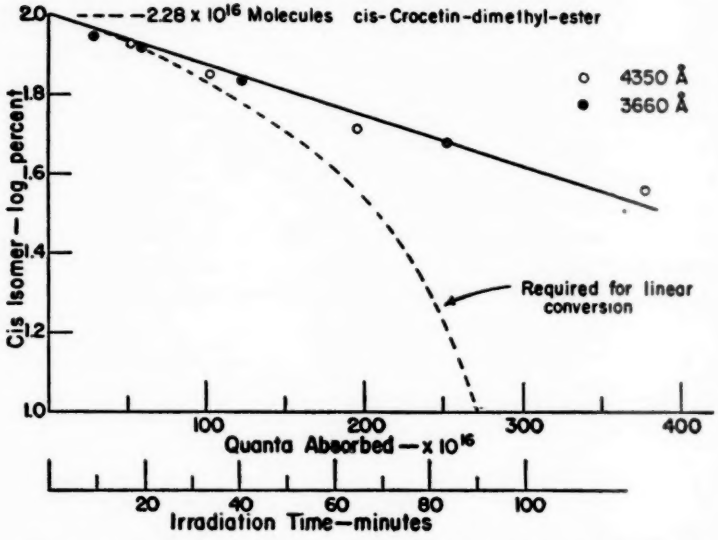


FIG. 3. The photochemical conversion of cis- to trans-crocetin-dimethyl-ester: an exponential function (solid curve) from physical data of Hausser and Kuhn and a linear function (broken curve) from biological data of Moewus (1938 et seq). The two functions are here equated at 10% conversion of the cis-isomer.

valences, as required by the reaction described by Moewus, are given in the last line of Table II.

Thimann (178) pointed out in 1940 that the alleged pattern of conversion of the labile precursor to stable end product could not be expected either on theoretical grounds or on the basis of the fundamental work on the cis → trans-crocetin-dimethyl-ester system of Kuhn and Winterstein (83). It is difficult to comprehend why a criticism of this magnitude, affecting a major aspect of the correlative mechanism, should have stimulated, during more than

a decade, neither reinvestigation of the disputed reaction nor any theoretical explanation of the unexpected linearity. All in all, the described linear transformation of precursor → end product, i.e., cis → trans-crocetin-dimethyl-ester, constitutes an exceedingly strange reaction and deserved from its authors greater recognition than was afforded by the casual description.

The complication of two contributing, but presumably independent, systems operative in the establishment of, and more particularly in the maintenance of, the necessary and critical ratios of cis- and trans-crocetin-dimethyl-esters in gamone activity has not been satisfactorily resolved. The final stage in the biochemical reactions leading to production of the two esters depends upon different enzyme systems for the two isomeric forms, those enzymes controlled by genes $gathe_{cis}$ and $gathe_{trans}$, respectively. The first of these systems is activated by visible light of all wave lengths, thus providing for formation of the precursor in light of any quality, whereas the latter system requires blue or purple light for its activation. Genetic constitution is thus held to determine the final cis/trans ratio, but, since the enzyme system controlled by the gene $gathe_{trans}$ can function only in blue or purple light, the trans isomer, under conditions conducive to copulation, is simultaneously produced in the medium by two distinct processes: *a*) the enzymatic conversion from crocin by the $gathe_{trans}$ system; *b*) the photochemical conversion of cis isomer, both present in the medium and the product of the current activities of the $gathe_{cis}$ system under the same conditions. Thus it would appear that under the required conditions for sexual activation the requisite and exceedingly critical cis/trans proportionality for cells of any valence would be difficult to attain and impossible to maintain. If the hypothesis of genetic control of cis/trans ratios is correct, photochemical conversion of the cis to the trans isomer can be only disadvantageous, since it limits the period during which a vital process of the plant is possible.

Moewus (119) cites the low quantum efficiency of the cis → trans conversion as evidence of the relative unimportance of the photochemical process in standardized experimental suspensions (containing 2×10^6 cells/ml.) where shading is appreciable; the difficulty, however minimized, remains. Much more data on the relative dependence upon light of these two separate processes

would be required to formulate a completely acceptable scheme of their interaction and of the actual mechanism which is operative in sexual activation and copulation.

One further area of uncertainty remains in the postulated gamone complex: the phenomenon of chemotaxis and its possible role in the mutual attraction of compatible gametes. As regards chemotaxis data, it would appear that the precise and critical cis/trans ratio differential required to cause chemotaxis can have no biological significance; the exact value rather reflects the resultant of numerous undefined physical conditions of the experiments. This conclusion necessarily follows from careful examination of the chemotactic experiments. A capillary containing a known ratio of cis/trans esters was introduced into a drop of gamete suspension containing the cis/trans esters in the specific ratio characteristic for the particular race and sexual strain; ratio differences $\geq 19/19$ resulted in the attraction of gametes to the tip of the capillary; smaller ratio differences showed no effect. Since the inevitable result of the diffusion of cis and trans isomers in one ratio into a much larger mass of water containing the same two substances in a different ratio is to effect a slight shift of the ratio in the gamete suspension, a ratio shift which falls in a steep gradient away from the capillary tip, the actual ratio change required to activate cells lying an appreciable distance from the tip must be tremendously less than 19/19. Insufficient data on relative volumes, concentrations, activation distances, capillary diameter, etc. have been furnished to allow an estimate of the minimal effective ratio change at the cell.

Even assuming a much lower chemotaxis threshold than postulated by the authors, the problem of a gamete in securing a mate, except by sheer accident, remains formidable. Consider what occurs when equal volumes of suspensions of cells of ♀4 (95/5) and ♀1 (65/35), for example, are mixed: the cis/trans ratio of necessity rapidly approaches a uniform value of 80/20. Furthermore, the solution would be homogeneous; initially no ratio gradients are possible and chemotaxis could not occur. Therefore, since gametes of these two valences do form clumps within a short time when mixed in this way, this homogeneity must be very transient. The processes, however, by which chemotactically effective gradients can be established on the basis of the postulated cis/trans

mechanism are obscure. Continued production of the two isomers in the two characteristic ratios by the gametes of the two valences is obviously essential, but consider the resulting limited possibilities: *a*) Secretion of a sedentary or sluggish gamete would shift the ratio of isomers in its immediate vicinity toward that of its own original filtrate; the secretion of each of two compatible gametes lying close together would tend to cancel out the effectiveness of that of the other, i.e., to dilute the ratio toward the background 80/20, and the shorter the distance between cells of opposite compatibility types, the more effective the cancellation. *b*) Actively motile gametes must leave behind the greater portion of their secretions and operate at or near the background 80/20, thus minimizing the opportunity for mutual attraction. *c*) Single sedentary gametes would establish steep short-range gradients about themselves, along which compatible gametes might be chemotactically attracted. The latter of these possibilities might account in certain cases for single pair formation, but it fails to account for group formation, since the resultant of the secretions of both types of gametes in the group would be only a change in concentration of total esters and not in ratio.

It thus appears unlikely that the differential cis/trans-crocetin-dimethyl-ester mechanism can adequately explain the observed phenomena of chemotaxis, mutual attraction and clustering. A different, or at least supplemental, mechanism, involving substances which have no effect upon the cells secreting them, would appear to be more consistent with known gametic behavior. Perhaps the substances responsible for atypical group formation, emphasized in the earlier papers as being secreted under the same conditions as the gamones but distinct from them in chemical properties and in physiological activities, are involved in these responses. Only one reference to these substances, a simple listing, has been seen in papers published since 1939 (108).

The contradictions and uncertainties set forth above constitute sufficient grounds for the gravest scepticism of the validity of the entire cis/trans mechanism as it has been described. The authors of the postulated mechanism, compounding too many improbable reactions based upon too little specific and unequivocal evidence for those particular reactions, make far too extensive claims as regards specifically detailed but unproved activities.

The authors throughout the course of the work appear to have ignored or neglected important details which fit uneasily into or contradict outright their major hypotheses. Two cases suffice to illustrate this tendency: *a*) The substances responsible for atypical group formation were described in 1933 as an integral part of the hormone mechanism. They have since been neglected in spite of the fact, according to their originally ascribed activities, that they must be co-existent and possibly co-active with the gamones, a portion of the mechanism which has received must attention. *b*) In 1940 (112), one of the authors cited the following experimental finding as evidence of degradation of a common precursor in the production of crocin and picrocrocin. Of 64 *mot*^{0} mutants, each incapable of producing crocin (the flagellar hormone), not a single one was capable of producing the substance which determined monoecious cells as ♀ (gynotermone). In work published since 1944, however, it has been equally unambiguously demonstrated that gynotermone activity resides principally, if not completely, in isorhamnetin, a product of a totally different biosynthetic series from that producing crocin and picrocrocin. Either the original observation was erroneous or the current interpretation is unwarranted.

Other inconsistencies of a lesser order of significance are more numerous than would appear justifiable: *a*) Two review papers published by Moewus within a year give 50,000 and 100,000, respectively, as the number of molecules of either isorhamnetin (gynotermone I) or 4-oxy-β-cyclocitral (androtermone I) required to determine the sexual type of monoecious cells. If these values can be ascertained with such exactitude, they at least deserve to be quoted consistently; if, as is most likely the case in the experimental determinations, there is no significant difference between the two values, the limits of accuracy of the determinations should somewhere be provided. *b*) In one of the recent reviews mentioned above, a table is given which lists the various hormones in *Chlamydomonas* and the minimal amounts of each which is required for activity. Two adjacent columns list for each hormone the minimal concentration showing activity and the number of molecules required per cell. The values for the number of molecules of crocetin-dimethyl-ester required per cell, calculated from the dilution factor given, is 2.5 times that shown; the calculated value for crocin is 14 times greater than the figure given; the values given in the two columns for the other hormones agree.

No substantial work on the *Chlamydomonas eugametos* group has been reported, so far as the reviewer is aware, from laboratories other than that of Kuhn and Moewus. Cultures of *C. eugametos,* however, have been made available since the war to several workers in this and in other countries, and it is confidently expected that independent investigations on the sexual mechanisms of this and interbreeding species will be forthcoming. Partial confirmation of the physiological aspects of sexual processes in planogametic algae may be inferred from the publications of Jollos (56), Geitler (39), Hartmann (45), Smith (175), etc. who have reported effects of filtrates of other species which were comparable to those so minutely explained by Moewus. The discovery in *C. moewusii,* by Lewin (90*a*), of mutant forms which produce flagella incapable of motility is consistent with the hormonal control of flagellation and motility as described by Moewus.

The current studies at Stanford University by Smith and his associates (175) on *C. Reinhardii,* etc. are of more than passing interest. In this species light has been shown to play an indispensable role in motility induction, clumping and sexual fusion. The behavior of the cells of this species to light, however, has been shown to be quite different from that described for *C. eugametos.* The major differences observed in *C. Reinhardii* are: *a*) the response to light, both as regards motility and ability to copulate, is much longer delayed; *b*) there is a definite correlation between light intensity and strength of the sexual reaction; *c*) ability to copulate in darkness, following flooding of the culture, is retained from previous illumination in the non-flagellated state; and *d*) light of various wave lengths, 4200–4600 Å, 5300–5700 Å, 5900–6400 Å and 6200–6800 Å, was equally effective in inducing copulation. The differences in detail of the responses to light of *C. eugametos* and *C. Reinhardii,* as reported by Moewus and Smith, respectively, probably only reflect species-specific variations of a common coordinative mechanism.

SEXUAL HORMONES IN PROTOSIPHON, BOTRYDIUM, MONOSTROMA. Moewus published, during the period from 1935 to 1940, in addition to the work on *Chlamydomonas,* a number of papers on similar, if less exhaustive, studies on three other algae. In these studies certain of the effects shown in *Chlamydomonas* were either anticipated or confirmed, but in no case was the work pursued beyond a descriptive study of the biological phenomena observed. These

studies will be treated in considerable detail here, since they are much less widely known than the more spectacular work on *Chlamydomonas.*

The earliest of these studies (100, 101) dealt with the influence of external conditions on sex determination in *Protosiphon botryoides,* a monoecious, isogamous, multinucleate, unicellular member of the Chlorococcales. In this species, as in monoecious strains of *Chlamydomonas,* gametes of a single culture copulate freely, but a number of residual unmated gametes is left in each culture. In different cultures of a single clone the residual gametes of a single clone are equally divided between the two mating types, arbitrarily designated as (+) and (−). Hydrogen-ion concentration, temperature and filtrates of residual gametes of either sexual type were shown to affect spectacularly the ratio of (+) to (−) gametes.

Vegetative growth on agar is normal and vigorous throughout the pH range 4.5–9.5. Plants were grown on agar at pH levels of 4.5, 7.0 and 9.5, following which cultures from each of these groups were induced to undergo gametogenesis by transfer to liquid media adjusted to pH values of 4.5, 7.0 and 9.5. Within the nine groups treated in this manner the ratio of (+)/(−) gametes varied from 100/0 in the 4.5 → 4.5 group through 50/50 in the 7.0 → 7.0 group to 0/100 in the 9.5 → 9.5 group. Other combinations of initial → final pH gave intermediate ratios in a pattern resembling a vector diagram of the two controlling treatments. The (+) mating type was defined as the type predominant in acid medium, and the (−) mating type as that predominant in alkaline medium.

Temperature was shown to have an almost equal effect on the determination of the sexual affinity of the gametes. In general, it was observed that a direct proportionality between temperature and the percentage of (+) gametes obtained but that the exact numerical ratio depended upon both temperature and hydrogen-ion concentration. Cultures grown on agar at pH levels of 4.5, 7.0 and 9.5 were subjected to eleven different temperature treatments comprising *a*) a pretreatment of the vegetative cultures at various temperatures for 15 hours, *b*) a second pretreatment of the vegetative cultures for short and varied lengths of time to more drastic temperatures, and *c*) varied temperatures during gametogenesis. The details of these treatments are given in Table IIIa. Thirty cultures were used for each treatment (a total of 990 cultures for the ex-

TABLE III

EFFECTS OF TEMPERATURE UPON SEXUAL DETERMINATION IN *Protosiphon*

a. Details of treatment

Treatment No.	Pretreatment I for 15 hours (°C)	Pretreatment II		Gametogenesis (°C)
		Duration (minutes)	Temp. (°C)	
1	7–8	60	–8	7–8
2	7–8	30	–8	7–8
3	7–8	10	–8	7–8
4	2	none		7–8
5	7–8	none		7–8
6	12	none		12
7	18–19	none		18–19
8	25–26	none		25–26
9	25–26	10	32	25–26
10	25–26	30	32	25–26
11	25–26	60	32	25–26

b. Results

Treatment No.*:		2	3	4	5	6	7	8	9	10
		Residual gametes—% total gametes								
Cultures and gametogenesis at pH:	4.5 (+)	68.1	68.0	69.9	73.0	77.0	84.0	94.0	94.0	97.0
	7.0 (+)	..	4.9	10.1	15.6	27.5	46.4	58.2	64.7	68.3
	7.0 (–)	67.7	64.2	61.0	50.3	32.5	19.6	11.3	5.3	..
	9.5 (–)	94.7	94.1	92.4	88.4	82.6	75.7	70.0	68.3	67.3

*Results of treatments 1 and 11 are not given. Presumably they are the same as those of 2 and 10 respectively.

periment), and from these were taken (presumably at random) ten samples which were transferred to hollow ground slides. Following the various treatments the cultures were allowed to incubate for six to seven hours to allow copulation to be completed. The quadriflagellate planozygotes are negatively phototactic, whereas any unfused gametes, termed " Restgameten " or residual gametes, are positively phototactic; this differential phototactic response afforded an easy and effective means of separation. From each slide culture a few residual gametes were tested as to sxeual type by matings with (+) and (–) gametes. The remainder of the gametes in each slide culture were then mated with gametes of the

appropriate sexual reaction. One to two days later the number of zygotes in the original culture was determined as well as that in a second slide in which the mating of the residual gametes from the first with the known tester type had occurred. From these data the relative frequency of (+) and (−) gametes in each culture and for each treatment could be calculated.

The results obtained in the 33 experimental lots thus treated are summarized in Table III. For cultures at any single level of hydrogen-ion concentration the frequency of (+) gametes relative to the total number of gametes increased with increased temperature. By varying both temperature and hydrogen-ion concentration, the relative frequency of gametes of the two mating types could be completely controlled. Maximum copulation, of course, occurred only in cultures in which the two mating types were present in equal frequency.

Moewus (98) had shown previously that (+) and (−) gametes of *Protosiphon* secrete sex specific substances which cause their mutual attraction in copulation. Filtrates of suspensions of gametes passed through a membrane filter retained their full activities. In the paper currently under discussion these filtrate effects were examined in greater detail. Filtrates were prepared from suspensions of both (+) and (−) gametes at pH levels of 4.5, 7.0 and 9.5. Cultures grown on agar at each of these pH levels were then brought to gametogenesis in each of the six filtrates from residual gametes. Fifty cultures were used in each of the 18 combinations.

The effectiveness of filtrates of suspensions of residual gametes in determining the mating affinities of gametes is shown in Table IV. In the two extreme cases, in acid (+) filtrate and in alkaline (−) filtrate, the mating type was determined to coincide completely with that of the gametes from which the filtrate was taken—with the result that in these cases no copulation occurred. Gametogenesis in all other filtrates, acid (−), neutral (+) and (−), and in alkaline (+), produced varying proportions of gametes of both mating types, and copulation occurred in all cases. In eight of these 12 combinations the residual gametes corresponded in mating type to that of the filtrate in which they were formed, while in each of the remaining four combinations both (+) and (−) residual gametes were found. In these four latter cases the hydrogen-ion level of the initial culture influenced the determination of mating affinity,

and the relative frequency of each of the two mating types which obtained in each case was the resultant of the two determining factors.

In a number of cases the effects of dilution of the active filtrates were determined. The greatest dilution at which complete determination occurred varied from 1:7 to 1:25 in the several cases, and in each case greater dilution resulted in copulation with the actual ratios of (+)/(−) gametes being determined by the pH of the diluted filtrate.

TABLE IV

EFFECTS OF PH UPON SEXUAL DETERMINATION IN *Protosiphon*

		Gametogenesis at pH:					
		4.5		7.0		9.5	
		(+) Filt.	(−) Filt.	(+) Filt.	(−) Filt.	(+) Filt.	(−) Filt.
Cultured at pH:	4.5	(100/0)*	30**/70	100/0	8/92	0/100	(0/100)
	7.0	(100/0)	0/100	100/0	0/100	0/100	(0/100)
	9.5	(100/0)	0/100	82/18	0/100	46/54	(0/100)

*Bracketed ratios: % (+) gametes/% (−) gametes, i.e., gametes of only one mating type produced, hence no copulation.

**Unbracketed ratios: $\frac{\%\text{ cultures with (+) residual gametes}}{\%\text{ cultures with (−) residual gametes}}$.

A further series of experiments with the two filtrates which completely determined mating type, acid (+) and alkaline (−), is of interest in that it shows the temporal requirements of the phenomenon of determination of sexual affinity. At room temperature (18–19° C), gametogenesis requires three hours, and determination of mating type must occur within the mother cell during this period, since the gametes are capable of copulating immediately after their emergence. Vegetative cultures were placed in acid (+) and alkaline (−) filtrates for periods ranging from 30 minutes to the full three hours required for gametogenesis, and each was then transferred to the filtrate of the opposite mating type.

The effects on mating type determination of each of the various treatments are shown in Table V. Less than 60 minutes in either

filtrate before transfer to the other had no effect, i.e., the mating type of all gametes was determined subsequent to one hour after initiation of gametogenesis. Exposure to either filtrate for 90 to 120 minutes partially determined mating type with the result that in each case copulation occurred and residual gametes were left in each culture dish. Determination of sexual affinity was complete after more than two hours exposure to either filtrate; the mating type of the gametes corresponded in all cases with that of the filtrate to which they had been exposed for 150 to 180 minutes, the transfer to the filtrate of opposite mating type having no effect on the gametes after initial treatments of these durations.

TABLE V

EFFECTS OF FILTRATES OF RESIDUAL GAMETES UPON SEXUAL DETERMINATION IN *Protosiphon*

(Tabulated from data of Moewus [100])

Time in acid (+) filtrate (minutes)	Transferred to alkaline (−) filtrate (in 25 cultures)	Time in alkaline (−) filt. (minutes)	Transferred to acid (+) filt. (in 25 cultures)
30	only (−) gametes	30	only (+) gametes
60	only (−) gametes	60	only (+) gametes
90	only (−) residual gametes	90	11 with (+) res. gametes 14 with (−) res. gametes
120	only (+) residual gametes	120	only (−) res. gamets
150	only (+) gametes	150	only (−) gametes
180	only (+) gametes	180	only (−) gametes

The equally interesting problem of the simultaneous effect of the filtrates in varying mixtures of the two was not investigated.

The control of sexual determination was further studied in *Botrydium granulatum,* a unicellular vesiculate member of the Chrysophyta. Moewus showed, in 1940 (111), that the sexual affinities of the gametes of this species, as in *Protosiphon,* could be determined completely during gametogenesis by the presence of filtrates of sexually active gametes. It was found that the filtrate from a suspension of gametes of a monoecious clone contained both gynotermone and androtermone and that these two hormones could be preferentially destroyed by physical and chemical means. Gynotermone was shown to be completely inactivated by irradiation with ultra-violet light, and a filtrate irradiated with U-V would determine all cells as ♂. The androtermone was shown to be

quantitatively destroyed by boiling the filtrate with 0.1 *N* H_2SO_4 for ten minutes; such a filtrate determined all cells as ♀. Vesicles undergoing gametogenesis in an untreated filtrate produced ♂ and ♀ gametes in approximately equal number. No attempts were made to identify the compounds active as gynotermone and androtermone.

Although the investigations on *Chlamydomonas, Protosiphon* and *Botrydium* overlapped in point of time and there was almost exact duplication of the superficial manifestations of the control of sexual determination between the members of the three genera studied, no mention has been made of any attempt to ascertain the degree of chemical similarities of the regulatory mechanisms of the three plants. Comparable intergeneric comparisons have been reported in two cases, both of which gave negative results. Active filtrates of *C. eugametos,* containing termones and gamones, were tried with *Dunaliella* and *Monostroma* by Lerche (89) and Moewus (110), respectively.

Yet another investigation of Moewus' deserves special attention, as it deals with a specific chemical effect involved in an earlier stage of the developmental sequence than those described above for *Chlamydomonas, Protosiphon* and *Botrydium.* In fact, it involves the controlled omission of a major portion of the life cycle. This work was done with *Monostroma wittrochii,* a dioecious form belonging to the Ulvaceae. The life cycle of this plant is like that of *Ulva* except for the reduction of the sporophytic generation to a small, uninucleate, single cell (104). At maturity the sporophyte produces 32 quadriflagellate swarmers, meiosis occurring in the first two nuclear divisions in this process. The quadriflagellate zoospores are thus haploid, one-half being (+) and the other half (−), but incapable of direct copulation. Each zoospore gives rise to a large thalloid gametophyte which produces biflagellate gametes of somewhat smaller size than the zoospores. Gametes from a single gametophyte do not fuse amongst themselves but fuse readily with those from a different and compatible thallus.

The genetic constitution of the two swarmer stages, both being haploid, must be identical, and the question arose as to the mechanism of differentiation of quadriflagellate zoospores and biflagellate gametes in the sporophytic and gametophytic generations, respectively. Could the environment in which the swarmers were pro-

duced determine their exact form? If so, was the determining environmental factor intracellular of extracellular? Could the morphological and physiological nature of the swarmers be affected by extracts or filtrates of sporophyte and/or gametophyte? A series of experiments gave explicit answers to most of these questions (110).

When sporophytes were induced to produce swarmers in a brei of gametophytic thallus, of either sexual strain, in sea water or soil extract, the characteristics of the swarmers were affected as follows: 64 were produced instead of the usual 32, they were smaller than ordinary zoospores, they were biflagellate instead of quadriflagellate, and they fused freely amongst themselves, the ratio of the two sexes being 1/1. A large increase in the number of sporophytes per unit gametophytic brei nullified all of these effects except the number of flagella, which for each swarmer was two. Furthermore, substitution of undamaged gametophytic thallus or filtrate from gametophyte for the brei gave no effect; the swarmers produced were normal zoospores in all respects. The effect on differentiation was shown to be unilateral: gametophytes placed in a brei of sporophytes in sea water produced only small biflagellate gametes which copulated normally with gametes from a gametophyte of the opposite sexual reaction.

The results obtained when a large number of sporophytes developed in a small volume of gametophytic brei suggested the possibility of important concentration effects of a constituent substance (or substances) of the gametophyte in determining the differentiative process in the sporophyte. A series of more elaborate experiments was performed in which were tested the effects of various dilutions of gametophytic extract as well as the effects of transferring the developing sporophytes from the diluted extract to other test solutions. The experimental procedures which were used and the results which were obtained in this series of tests are summarized in Table VI.

Comparison of the first four treatments listed in Table VI shows that with dilutions of the gametophytic extract the altered characteristics of the swarmers successively revert to those of the normal zoospores as higher dilutions are used. The results of treatments *e*, *f* and *h*, as compared to those of *b*, *c* and *d*, show that the ability to copulate is determined subsequent to the initiation of division of

the protoplast. The number of flagella, however, appears to be determined before cellular division, *e* and *f* vs. *h*, except where sporophytic extract was added at the time of the initiation of cellular division, in which case the swarmers were quadriflagellate, *g*. This reversal of a character previously determined would appear to be a specific activity of the extract of the sporophyte, since the extract of the gametophyte under the reversed conditions obtaining in treatment *h* had no corresponding effect on the number of flagella. The swarmers formed under treatments *e* and *f*, each

TABLE VI

THE CONTROLLED SUPPRESSION OF THE GAMETOPHYTIC GENERATION IN *Monostroma*

(Tabulated from data of Moewus [110])

Treatment	No. of swarmers	Size	No. of flagella	Copulation**	
				Within the group (% copul.)	With normal gametes (% copul.)
a. 5 sporophytes in E*	64	S	2	100	100
b. 5 sporophytes in E/2	32	L	2	0	100
c. 5 sporophytes in E/4	32	L	2	0	0
d. 5 sporophytes in E/10	32	L	4	0	0
e. 2 sporophytes in E/2→E†	32	L	2	40 ($e \times f = 100$)	100
f. 2 sporophytes in E/4→E	32	L	2	40	100
g. 2 sporophytes in E/4→ extract of sporophyte	32	L	4	0	0
h. 4 sporophytes in E/10→E	32	L	4	2–100 2–0	100

*E = extract of 10 cm.^{2} gametophytic thallus in 5 ml. of sea water.

**Zygotes resulting from all fused swarmers produced normal sporophytes.

†Transferred from the initial solution at the time of initiation of cellular division.

group showing only incomplete intragroup copulation, fused freely when mixed together.

The various effects of the extract of gametophytic thallus on the characteristics of the swarmers were quite specific. None of the effects could be duplicated by any of the several factors previously shown to have spectacular influence on the behavior of gametes of *Protosiphon, Chlamydomonas* and *Botrydium*. These included temperature changes, hydrogen-ion concentration, filtrates of gametes containing gamones and termones, and concentration of sea water.

The active constituent of the gametophytic extract was shown to be non-dialysable and to be destroyed by a temperature of 100° C for five minutes. It retained its full activity, however, when the extract was added to an equal volume of absolute alcohol, evaporated to dryness at 30° C and then re-dissolved in water. The active compound is in all probability a protein. The obvious difficulty of a protein controlling a regulatory mechanism, the manifestations of which are entirely intracellular, such as that described for *Monostroma,* was recognized by the author, but no hypothesis was advanced to account for the probable means of its achievement. The lack of final knowledge of the mechanism involved, however, does not detract from the significance of a work which points toward a better understanding of morphogenetic phenomena in the lower plants.

In considering these many cases of hormonal activity in the algae, it is of parenthetical interest that such a correlative mechanism has not as yet been found in any fungus having gametic copulation, although the similarity between formation, morphology and behavior of planogametes in aquatic fungi and algae would indicate a common type of regulatory mechanism in the two cases. That the correlative mechanism has not been found in fungi possibly results from the fact that in none of the forms reproducing by differentiated gametes is there sexual dimorphism coupled with the capacity of indefinite vegetative propagation; it is therefore impossible to secure large clonal masses of fungal planogametes of known sexual affinities. The discovery, or creation in the laboratory by means of genic mutations, of heterothallic strains of *Allomyces,* etc. would in all probability be shortly followed by description of a hormonal coordinating mechanism in planogametic fungi.

SEXUAL HORMONES IN GAMETE-GAMETANGIAL COPULATION

Coordination of sexual processes by means of sexual hormones has been demonstrated in a number of species characterized by gamete-gametangial copulation. The demonstrated cases, however, apply to the members of only two groups of plants, both fungi, the Saprolegniales of the aquatic Phycomycetes and the Sphaeriales of the pyrenomycetous Ascomycetes; no comparable correlative mechanism has been shown in any alga exhibiting this basic type of sexual fusion.

In contrast to the apparent similarity of correlative phenomena found throughout the algae having planogametes discussed above, there is no evidence of a single pattern of coordination obtaining in different groups of fungi which are widely dissimilar phylogenetically. That this should be the case is not too surprising in view of the great dissimilarity of the basic patterns of sexuality in the two groups of fungi represented in this category: control of mating type by sexual factors per se (Saprolegniales) vs. control of mating type by incompatibility factors superimposed upon hermaphroditism (Sphaeriales). The evolutionary histories of the two groups would appear to have been exceedingly divergent.

Historically the Saprolegniales are of considerable interest as regards the growth of the concept of chemical regulation of sexual processes in plants. De Bary, as early as 1881 (30), pointed out for a species of *Saprolegnia* the probability of the initiation of antheridial hyphal production and of the attraction of antheridial hyphae to the ripening oogonial initials as dependent upon the secretions of specific chemical entities by the oogonial initials. This view was later upheld by Kaufmann (57), based on observations on *Saprolegnia hypogyna,* a form in which oospores normally develop parthenogenetically, antheridia being produced only rarely. Under the influence of certain inorganic salts, however—phosphate and nitrate were considered chiefly responsible—antheridial hyphae could be induced in profusion on this species. These inorganic salts were considered necessary for elaboration of the oogonial secretions postulated by De Bary.

Couch (28), in an intensive study of heterothallism in *Dictyuchus,* published shortly after the first unambiguous demonstration by Burgeff in 1924 of sexual hormones in plants, included the results of a study designed to demonstrate the activity of sexual hormones in this genus. The following observations on *Dictyuchus monosporus* were interpreted as indicative of hormonal coordination: production of antheridial hyphae and oogonial initials on reacting ♂ and ♀ mycelia, respectively, at locations far removed from the line of contact; sexual interaction between the ♂ strain of *D. monosporus* with *Thraustotheca primoachlya* (homothallic) in intergeneric mating; and directional growth of antheridial hyphae to ripening oogonial initials.

The following experiments typify those designed and performed

by Couch in his attempt to demonstrate sexual secretions: *a*) many ♂ plants placed in a shallow layer of water but separated from a single ♀, to test the secretion by the ♂ of substances inducing oogonial formation; *b*) three mated pairs of ♂'s and ♀'s located in a shallow layer of water but separated from two single ♀'s, to test the possibility that sexual hormones were secreted only by ♂ and ♀ hyphae in actual contact; *c*) physical separation of ♂ and ♂ plants in water by means of a permeable collodion membrane, in an attempt to demonstrate trans-membrane activation; and *d*) addition of a brei of plants of each sex to cultures of plants of the opposed sexual sign. The results in all cases were negative. This does not, however, detract from the importance of the work, for both the observational evidence for hormonal coordination and the methods of experimentation which were first used here have more lately abundantly proved their validity in work on a closely related genus, *Achlya*.

The failure of these efforts to give positive results is explicable in terms of subsequent findings in closely related forms. The experiments were obviously designed on the assumption, commonly held since the earliest descriptions of the sexual process in water molds, that oogonial initial production initiates sexual reaction. Since the initiating reaction definitely occurs not in the ♀ but in the ♂ (15, 136, 137), choice of plants of the opposite sexual signs in the first two experiments would in all probability have given positive results.* A further important characteristic of heterothallic water molds, not fully appreciated until somewhat later (15, 85, 138), is the occurrence of a wide variety of sexual strains including many sexual intergrades which differ greatly in sexual potency. Only strongly sexed ♂'s and ♀'s secrete sexual hormones in sufficient quantities to induce reactions at significant distances from the loci of secretion. Even so, the membrane matings, which were made in water, would probably have given positive results had the matings been made in agar, thereby restricting the dissemination of sexual secretions to diffusion alone. Finally, it is

* The recently published textbook, "Morphology and Taxonomy of Fungi" by Bessey (11), furnishes a prime illustration of the manner in which inaccuracies of this kind are carried along in successive texts. This particular case, however, is distinguished by an uncommon twist: the author, in addition to his recapitulation of the original and erroneous description of the initiation of the sexual reaction in the saprolegniaceous fungi, misquotes in its support the specific papers which contained its refutation.

now known that the hormones of water molds which initiate sexual reaction occur almost entirely in the liquid in which the plants are submerged; only a negligible amount can be extracted from the mycelium.

The first demonstration of sexual hormones in fungi characterized by gamete-gametangial copulation was provided by Bishop (14) for *Sapromyces reinschii* in his doctoral dissertation in 1937; publication of this work (15), however, was delayed until the year 1940. In connection with a study of heterothallism and sexuality in *S. reinschii,* Bishop gave unequivocal proof that secretion of ♀ plants induced the production of antheridial hyphae on the ♂. In matings of ♂ and ♀ plants on an agar medium, production of antheridial hyphae on the ♂ prior to contact with the ♀ was frequently observed, and it was regularly the case that antheridial hyphal development was more intense upon that portion of the ♂ mycelium proximal to the ♀ than elsewhere upon the ♂ mycelium. Production of antheridial hyphae on ♂ plants grown sterilely upon an agar medium could be regularly induced by addition of filtrate, freed of spores and hyphal fragments by passage through a Berkefeld filter, of ♀ plants grown sterilely in water. No response was observed when the reciprocal test was performed, i.e., ♀ plants in ♂ filtrate. Oogonial initials were occasionally formed on ♀ mycelia before contact with ♂'s when mated upon a semisolid medium, and antheridial hyphae were observed always to grow directly to the exact distal ends of oogonial initials. Production of oogonial initials and the directional growth of antheridial hyphae were considered to result from the activities of diffusible substances, but no specific experimentation was performed to test the validity of this assumption.

Bishop's summation, " that some substance diffusing from the ♀ mycelium stimulated the development of antheridial hyphae on the ♂ and that these in turn were influenced to grow directly toward the oogonia by some substance diffusing from the oogonia themselves ", was confined to only two of the many succeeding phases of the sexual reaction. No attempt was made to postulate the minimal hormonal mechanism which would integrate the observed phenomena. It is unfortunate that the work on chemical correlation in *Sapromyces* has not been continued beyond these preliminary findings.

THE HORMONAL MECHANISM IN ACHLYA. The hormonal mechanism regulating the sexual process has been more completely worked out by Raper and associates (135–137, 139–143, 145) in two heterothallic species of the saprolegniaceous genus *Achlya*, *A. ambisexualis* and *A. bisexualis*, than elsewhere in the fungi. The entire sexual process, up to and including differentiation of ♀ gametes (oospheres or eggs), in these forms has been shown to

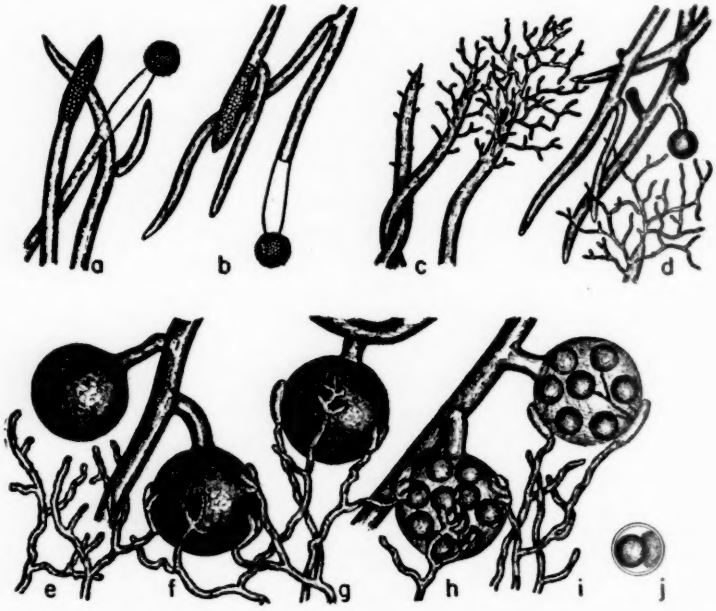


FIG. 4. Sequence of morphological changes in the sexual interaction between male and female plants of heterothallic *Achlya* (from Raper, 1951).

be coordinated by specific diffusible hormones, each successive stage in the reaction being initiated and precisely controlled by the secretions of the last preceding stage.

Five major stages, sequentially invariable and morphologically distinct (Fig. 4), are recognized as comprising the sexual reaction in *Achlya* (136): i) production of antheridial hyphae on the ♂ plant (c), ii) production of oogonial initials on the ♀ (d), iii) directional growth of antheridial hyphae to the oogonial initial (e)

and, following actual contact of the tip of the former with the wall of the latter, delimitation of the antheridium by formation of a septum across the antheridial hypha (f, at right), iv) delimitation of the oogonium by basal septation (g) and reorganization of the protoplasmic contents of the oogonium to form a number of uninucleate spherical oospheres (h), and v) transfer of ♂ nuclei, through tiny tubes extending from the antheridium into the lumen of the oogonium, to the several oospheres (i). Four specific hormones are now known to be involved in the initiation and quantitative control of the first stage (141), and a minimum of one hormone is known to be essential for each of the next three stages (137). Although the final stage, fertilization, may also be hormonally controlled, no means of direct experimentation has yet been devised to test this possibility.

Strong indicative evidence of chemical coordination of the sexual process in *Achlya* follows from a number of abundantly confirmed observations: *a*) The strict sequential pattern of the various phases of the sexual reaction indicates an absolute stage-to-stage dependence; the successive secretion of stage-characteristic specific substances is the most feasible means of assuring the observed stage-to-stage dependences. *b*) The close correlation between the intensity of the sexual reaction and the temporal progression of the reaction indicates a rigid quantitative, as well as qualitative, stage-to-stage dependence most easily explained by chemical regulators. *c*) Complete disruption of the sexual process at any desired stage by manipulation of the chemical composition of the medium indicates immediate products of the plants' metabolism as the agents of sexual correlation. *d*) The pattern of sexual reactions in interspecific and intergeneric matings, ranging from no response through all degrees of partial compatibility to complete compatibility, indicates a common basic mechanism of sexual coordination involving minor species-characteristic differences, probably chemical in nature (136, 142).

Conclusive evidence for a hormonal mechanism follows from the distant or telomorphic (23) induction of all stages of the sexual process. Telomorphic reactions may be easily induced under various circumstances, either by use of filtrates or by physical separation of mated plants by permeable membranes (137, 139–142, 145).

Integration of information from telomorphic reactions, induced in various ways, has been the chief means of ascertaining the basic characteristics of the hormonal mechanism: the minimal number of hormones involved, the loci of their secretion and the specific activities which they induce. Because of the prime importance of these reactions in this work, a few of the more basic means of inducing them are briefly described.

When mated in water, two strongly compatible plants, each growing upon a halved hemp seed, react vigorously along the line of intermingling of their mycelia. In addition to this localized activity, however, limited reactions are induced on both ♂ and ♀ mycelia at points far removed from the line of contact: a peripheral fringe of antheridial hyphae is produced on the ♂, and scattered oogonial initials develop over the entire ♀ mycelium (137).

Strong ♂ and ♀ mycelia, inoculated at opposite sides of a plate of semisolid medium, are strongly and mutually activated prior to contact of the two radically expanding mycelia. This reciprocal interaction occurs in two distinct stages: production of antheridial hyphae on the ♂ and of oogonial initials on the ♀. The first reaction may occur when the two mycelia are separated by as great a distance as 18 mm.; the second reaction, initiated only after the first has been well established several hours, occurs at greatly reduced intermycelial distances. Oogonial initial proliferation (see below) commonly results when oogonial initials are produced prior to contact of the mycelia of the two strains (137, 142).

The mating interactions clearly attributable to diffusible hormones may be better observed, however, by interposing a permeable cellophane membrane between the two plants mated on a suitable semisolid medium (137, 142). Under these conditions the interaction between strongly compatible ♂ and ♀ strains is commonly intense and proceeds normally through most of the stages of the sexual process. The interaction does not proceed, of course, to zygote formation; the actual end-point achieved in any case depends upon the particular strains used in the mating. In matings between fully compatible strains the reaction commonly stops with differentiation of antheridia, closely appressed to the membrane, but in one species, *A. bisexualis*, the reaction sometimes proceeds through delimitation of the oogonium and differentiation

of oospheres *. In this species, at least, it has thus been shown that the entire sexual progression is under hormonal control and that physical contact between the two mated mycelia is required only for the transfer of ♂ nuclei in fertilization.

Two important relational effects are more strikingly shown in membrane matings than by any other means: *a*) In the absence of compatible ripened oogonial initials, antheridial hyphae in contact with a definitive surface continue to elongate as though they lay free in the medium. Following the ripening of compatible oogonial initials in the immediate vicinity, however, antheridial hyphae, wherever they come into contact with the membrane, produce at their tips characteristically branched swellings which become delimited as antheridia. Antheridial formation and differentiation are thus shown to require both contact with a definitive surface and specific secretion(s) of ripened oogonial initials. *b*) Oogonial initials remain unchanged several hours after attainment of maximal size. If they are not reached by antheridial hyphae during this period—the inevitable case in membrane matings—a renewal process is initiated. One to several new oogonial initials grow out of the original and rapidly enlarge; if these are not reached by antheridial hyphae during their period of ripeness, they too may in turn proliferate to form a new crop of initials. The proliferative rejuvenation of oogonial initials is also frequently observed in contact matings between partially compatible plants where antheridial hyphae reach the oogonial initials. Such rejuvenation occurs in some cases in which antheridia have been delimited at the tips of antheridial hyphae in contact with the initial. Thus inhibition of oogonial initial proliferation, induction of oogonial delimitation and reorganization of the oogonial protoplasm in oospheric formation

* No detailed description of this reaction has heretofore been published. In matings between strong ♂ and ♀ strains of *A. bisexualis*, in which the membrane is considerably wrinkled, occasional oogonial initials, lying within narrow folds and very close to but not necessarily in contact with the membrane, thus practically surrounded by a continuous layer of differentiated antheridia, become delimited and produce apparently normal oospheres. Only small oogonial initials, producing at differentiation only three to five oospheres, appear to be affected, no large delimited oogonia ever having been observed under the same conditions. The differentiated oospheres persist seemingly unchanged for a period of two to three days, after which they rapidly disintegrate.

appear to be coordinate processes, successively dependent upon secretion(s) of contiguous compatible antheridia.

A still different method of inducing telomorphic responses is through the use of filtrates and filtrate fractions of vegetative plants and of sexually activated plants. This method has more far-reaching potentialities than those listed above, since single responses can be induced either in ♂ or ♀ plants in the absence of plants of the opposed sexual sign, thus permitting quantitative examination of the isolated reactions. Quantitative studies using filtrates have been limited to date to investigation of the factors controlling the initial stage of the sexual reaction, production of antheridial hyphae (see below). Qualitative filtrate studies of later stages in the sequence, however, have furnished much useful information; the following example illustrates one such study.

A perfusion technique, in which a slowly flowing stream of water was passed successively over a number of plants in micro-aquaria, was used to establish the order and origin of secretion of the basic types of hormones. For example, a series of plants in the order ⟩—♂1—♀2—♂3—♀4→, in respect to direction of the flow, showed no effects on the plants in the first two vessels but gave intense production of antheridial hyphae on the ♂ in the third vessel and oogonial initial production on the ♀ in the fourth. When the order was changed to ⟩—♀1—♂2—♀3—♂4→, intense production of antheridial hyphae resulted in aquaria 2 and 4, oogonial initial production in aquarium 3, and strong attraction of antheridial hyphae in vessel 4 to the tip of the capillary connecting this vessel with aquarium 3. Summing the results of these two series, it is clear that: *a*) initiation of the entire sexual reaction depends upon secretion by the ♀ of an agent(s) which induces production of antheridial hyphae on the ♂, *b*) production of oogonial initials on the ♀ depends upon a substance(s) secreted by the ♂ only after its activation by the ♀, *c*) attraction of antheridial hyphae depends upon a substance(s) secreted by the ♀ only after the production of oogonial initials in response to the secretion(s) of the sexually activated ♂, etc. These results are easily confirmed by use of corresponding filtrates from which spores and viable hyphal fragments have been removed by passage through a bacteriological filter (137).

From the results of the various lines of investigation briefly de-

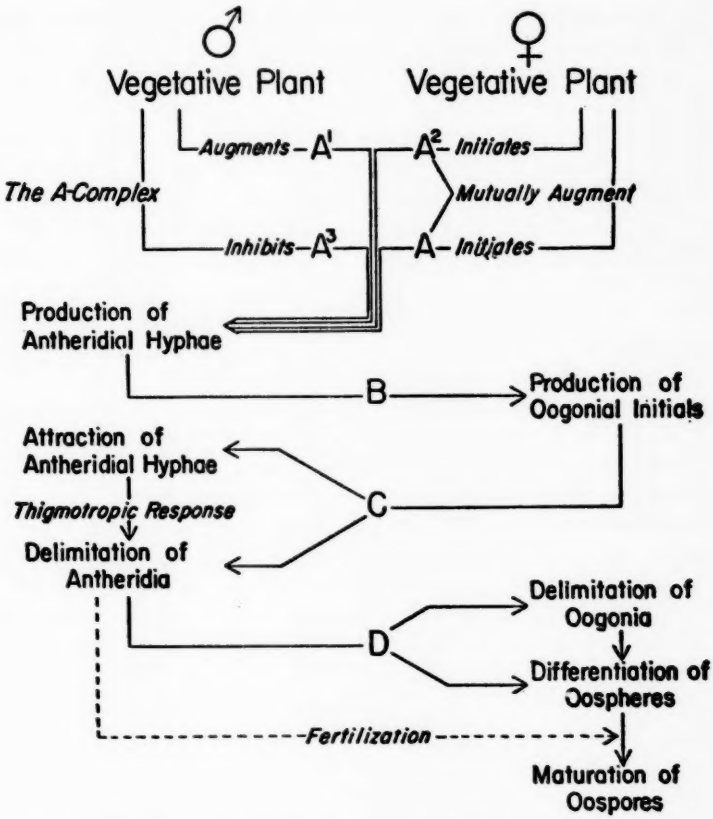


FIG. 5. The hormonal mechanism which coordinates the sexual interaction between male and female plants of *Achlya* (from Raper, 1951).

scribed above it has been possible to formulate the minimal mechanism operative in initiating and coordinating the sexual processes in *Achlya*. A diagrammatic scheme of the hormonal mechanism is given in Fig. 5. This scheme has passed through several revisions since its first publication in 1939 as additional hormones and hormonal activities have become apparent in continuing work, and it is probable that it is, as presented here, largely skeletal, particularly as regards those stages subsequent to the production of antheridial hyphae.

The initial reaction, production of antheridial hyphae on the ♂, is induced and quantitatively regulated by four distinct hormones, collectively designated the A-complex. The A-complex comprises hormones A and A^2, secreted by the vegetative ♀ plant, and hormones A^1 and A^3, secreted by the vegetative ♂. Hormones A and A^3 are acetone- and dioxane-soluble, whereas hormones A^1 and A^2 are acetone-insoluble and water-soluble. The two hormones secreted by vegetative plants of each sexual strain are thereby readily separable and may be tested separately or in any desired combination.

Production of antheridial hyphae on the ♂ constitutes a sensitive and reliable biological assay for the hormones of the A-Complex (139–141, 145). Four-day-old ♂ plants, growing upon halved hemp seed in water, show a discernible response within an hour when placed in water containing the appropriate hormones in the proper concentrations. Upon the vegetative hyphae there appear, de novo, numerous small lateral bumps, each of which by rapid elongation develops into a thin contorted filament, the antheridial hypha. By the end of four hours the antheridial hyphae commonly have attained a length of 0.2–0.5 mm. and may be easily counted. The relative abundance of antheridial hyphae is an indication of the intensity of the reaction. Each hypha is considered as an individual, and the average number of antheridial hyphae per three mm. vegetative hyphal tip has been adopted as a convenient index of reaction intensity. Depending upon the degree of accuracy required, 20–100 hyphal tips, taken at random on two or more mycelia, are counted and averaged for each test. The average number of antheridial hyphae can be varied at will from 0 to about 75 by alteration of the hormonal content of the test solution.

The qualitative activities of the hormones of the A-Complex are as follows: hormones A and A^2, secreted by the ♀, singly or together induce the formation of antheridial hyphae on the ♂; hormone A^1, secreted by the ♂, significantly augments the activities of hormones A and A^2, while having no inductive activity of its own; and hormone A^3, also secreted by the ♂, acts to depress the response induced by the other hormones.

The pattern of quantitative control of antheridial hyphal production by the four hormones of the A-Complex, however, is considerably more complicated. Each of the four hormones affects

the response in a characteristic manner, and their effects in combination are not always predictable from their separate activities. Thus the average number of antheridial hyphae produced per three mm. hyphal tip is: *a*) a logarithmic function of concentration of hormone A, *b*) a linear function of concentration of A^1 in the presence of A, *c*) an exponential function (of the type $y = e^{ax^k}$) of the concentration of A^2, *d*) roughly a linear function of the concentration of A^2 in the presence of A, *e*) increased by A^1 in the presence of A^2 alone or in the combined presence of A and A^2, and *f*) decreased by A^3 in the presence of A or A plus A^2 (141).

Production of antheridial hyphae is affected markedly also by a number of other factors, including temperature, hydrogen-ion concentration, electrolyte concentration and the presence of nutrients. In the biological assay for the A-Complex hormones, these factors must be rigorously controlled (139).

A further point of interest and significance is the pattern of secretion of hormone A^1 by the ♂ plant (139, 140). While this hormone alone can not induce production of antheridial hyphae, it greatly augments the activity of the ♀-secreted hormones, and it has been clearly demonstrated that the plant secretes sufficient quantities of the hormone during the four-hour test period to affect significantly the reaction. Furthermore, hormone A^1 is secreted in variable quantities in relation to time so that the apparent sensitivity of the ♂ plant to ♀-secreted hormones varies rhythmically through a 19 to 20 hour cycle. All efforts to determine whether hormone A^1 is essential to the reaction have been unsuccessful; a number of experiments designed to test the essentiality of the hormone have given ambiguous results. Hormone A^1 secretion need not interfere with assaying of the other hormones, however, since its activity, when added in optimal concentration, is predictable (141).

The antheridial hyphae secrete, during the period of their formation and continued growth, a substance, hormone B, which specifically induces formation of oogonial initials on the ♀ mycelium. Although the quantitative control of oogonial initial production has not yet been worked out, it is quite apparent that such control is operative: the intervening time between production of antheridial hyphae and of oogonial initials is inversely proportionate to the intensity of the former reaction, while the number of oogonial

initials ultimately produced varies directly with the intensity of the response of the ♂. These relationships obtain in all matings of strongly compatible strains; the exceptions which have been observed are explicable in terms of hormonal specificities.

The oogonial initials secrete hormone C during the period of their rapid enlargement and for some time after maximal size has been attained. Hormone C induces and controls two specific responses in the antheridial hyphae of the sexually activated ♂: *a*) it causes the positive tropic growth of antheridial hyphae along its concentration gradient to the source of its secretion, the oogonial initial, and *b*) it evokes the formation and delimitation of the swollen branched antheridium when an antheridial hypha comes into contact with a definitive surface, be it an oogonial initial wall or a cellophane membrane. The thigmotropic stimulus appears to be essential in the differentiation of the antheridium, since delimited antheridia have never been observed lying free in the medium.

The antheridia, following their delimitation, secrete yet another substance, hormone D, which is essential for final development of the oogonium. Hormone D, like hormone C, induces two specific responses: *a*) delimitation of the oogonium by formation of a septum across the oogonial stalk; *b*) reorganization of the protoplasmic contents of the oogonium to form a number of uninucleate oospheres, or eggs. The usual presence of delimited antheridia upon the oogonium at the time of the final differentiation might appear to indicate a thigmotropic response similar to that involved in antheridial development; the exceptional case of oosphere formation in the absence of contiguous antheridia in *A. bisexualis*, as described above, argues against this view. Inadequate concentration of hormone D, in all other cases where delimited antheridia are withheld by a membrane barrier, would more plausibly explain the observed facts.

Hormones C and D, each of which induced two characteristic and distinct responses, may well consist of complexes of hormones instead of single substances. This possibility is enhanced by the fact that in many interspecific matings the first response in each case is not invariably followed by the second. Conclusive proof of the dual nature of these hormones, however, is lacking.

Thus initiation and correlation of the sexual processes in heterothallic species of *Achlya* are seen to depend upon a minimum of

seven distinct and specific hormones, four secreted by the ♂ and three secreted by the ♀. By means of these chemical agents each stage in the sexual progression is directly and completely dependent upon the immediately preceding stage. Consummation of the entire sexual interaction, i.e., formation of zygotes, is therefore prevented by failure of any of the several component stages. By proper choice of experimental procedure the sexual reaction of these plants may be disrupted at any desired stage.

The chemical identities of the various hormones are unknown. An attempt was made to isolate and identify hormone A of *A. bisexualis* ♀ (145). Although neither of these objectives was attained and the active substance could not be assigned to a specific group of organic compounds, many of its properties were determined and tremendous concentration of the hormone was achieved. A final fraction, weighing 0.2 mgm. and still impure, contained 37% of the hormone A activity initially present in 1440 liters of filtrate from ♀ plants and induced the production of antheridial hyphae on ♂ plants when tested in a concentration of 10^{-13}. Aside from a few preliminary studies on hormone A^1, no further chemical work has been attempted. A major obstacle to the isolation and identification of these hormones is the tremendous quantities of raw material required, quantities which cannot be feasibly grown in the facilities available in academic laboratories.

It has recently been demonstrated that many homothallic species of *Achlya* and of at least one closely related genus, *Thraustotheca,* have sexual hormonal mechanisms similar to and interactive with that of the heterothallic species described above. This demonstration (142) depended upon the reactions elicited in interspecific and intergeneric matings of homothallic plants with ♂ and ♀ strains of heterothallic species, upon the reactions of homothallic plants to filtrates and filtrate fractions of ♂ and ♀ heterothallic plants, and upon the reactions of heterothallic plants to homothallic filtrates.

Interspecific and intergeneric matings between homothallic and heterothallic plants revealed all shades of reaction between no reaction, with neither mate affected, to complete compatibility, with formation of normally matured oospores (zygotes). In most cases, however, definitive responses were elicited in one or both mates, but the reaction failed in each case to proceed beyond a stage in the progression characteristic for the particular contrast. Such

failures recall the " imperfect hybridization " of Blakeslee (16) and are here interpreted as due to hormonal specificities. Two instances in which the sexual process was consummated in interspecific and intergeneric matings, *A. americana* and *T. clavata* × *A. ambisexualis* ♀, respectively, the coordinative mechanisms of the mated plants being completely interactive, prove the basic similarity of hormonal control in homothallic and heterothallic species.

Quantitative studies of the production of antheridial hyphae induced in homothallic and heterothallic plants by filtrates and filtrate fractions derived from plants exhibiting both types of sexuality, however, revealed striking dissimilarities of the hormones of the A-complex actually involved in the two types of plants. One example will serve to illustrate such differences. Hormone A of *bisexualis* ♀ alone induces in *americana* (☿) a response which is quantitatively indistinguishable from that induced by the same concentration of hormone A in *ambisexualis* ♂ in the presence of an optimal concentration of A^1; addition of A^1 to *americana* has no significant effect. Similarly, hormone A^2 of *bisexualis* ♀ alone induces in *americana* effects equivalent to those induced by hormones A^2 and A^1 in *ambisexualis* ♂ ; in this case, unlike in that preceding, addition of hormone A^1 inhibits the response of *americana* to hormone A^2. *A. americana* produces no secretion having activity similar to that of A^1.

It is concluded from these studies that similar basic mechanisms correlate sexual processes in homothallic and heterothallic species, but that, although in most cases the two are interactive to a greater or lesser degree, they differ greatly in detail. Hormonal specificities and a general simplification of the hormonal mechanism in homothallic species, as compared to that of heterothallic species, would account for the observed differences.

Certain other hormonal effects in interspecific and intergeneric associations involving homothallic and heterothallic species are of significance. Hormone A of *A. bisexualis* ♀ and *A. ambisexualis* ♀, in addition to inducing very intense production of antheridial hyphae in *T. clavata, A. americana* and in most other homothallic species, completely suppresses the formation of oogonial initials on the homothallic species as well as causes the ♀ initials already formed to degenerate or to become redifferentiated as antheridial hyphae. Thus in the presence of a strong heterothallic

♀ plant, the normal homothallic plant is transformed into one which can function only as a male. Similarly, hormone A^1 of *A. ambisexualis* ♂ completely suppresses the production of oogonia on *T. clavata* and two unidentified homothallic species of *Achlya*. In this latter case normal homothallic plants in close association with strong heterothallic ♂ plants are reduced to sexual impotence. Such demonstrated activities of sexual hormones largely explain the numerous cases of sexual anomalies in the water molds described by Humphrey (54), Maurizo (96), Coker (24) et al. and indicate certain problems of possible ecological interest.

THE NEUROSPORA CONTROVERSY. Among the Ascomycetes characterized by gamete-gametangial copulation, the presence and activity of sexual hormones have been suspected in a number of species and conclusively demonstrated in very few. In none of these cases, however, has an integrated hormonal mechanism been described.

In 1931 a paper by two French workers, Moreau and Moruzi (122), first claimed demonstration of sexual hormones in a species belonging to the Ascomycetes and incidentally touched off an interesting controversy. Two strains of a heterothallic form of *Neurospora,* strain M, isolated from a decaying mushroom, and strain N, obtained from Prof. B. O. Dodge, were inoculated into the two ends of a U-tube. In single culture each strain produced only small sclerotia. In the U-tube matings, sclerotia, somewhat larger than normal for the single culture, were produced on the mycelium of the N strain, while mature perithecia were produced on the mycelium of the M strain. Small sections of the connecting tubes containing agar were cut out and incubated under moist conditions; that no growth resulted was considered proof that there had been no hyphal growth between the two ends of the tube. The conclusion was reached, therefore, that the production of perithecia was the result of the action of a diffusible hormone(s) passing from one mycelium to the other through the solidified medium in the connecting arm of the tube.

Later in the same year Dodge (33), skeptical because of the possible confusion of (sterile) sclerotia for perithecia, repeated the U-tube experiments, using one conidial strain and one non-conidial strain. His results in each case were negative, perithecia appearing in the ends of the tubes only when air spaces, formed by the shrinking of the agar medium, were present throughout the greater

part of the length of the connecting tube. In such cases where perithecia were formed it was shown that both strains, conidial and non-conidial, were present in the medium in the immediate vicinity of the perithecia.

A year later the French workers (123) published certain observations purporting to add further evidence of hormonal action at a distance in perithecial induction. A wide restraint area in the region where compatible mycelia intermingled and the two lines of perithecia on either side of this area, their chief new evidence, had been observed by Dodge some 20 years earlier in *Ascobolus*.

In the following two years two papers by Aronescu (2, 3) subjected the hormone theory to intensive genetical analysis. Using compatible conidial and non-conidial strains and the U-tube technique, asci were isolated from perithecia whenever they appeared, and the sexual and conidial characteristics of the mycelia derived from the eight separated ascospores of each were determined. Theoretically, according to the author, three possibilities existed: *a*) segregation of both factors in a perfectly normal Mendelian ratio if nuclei were transferred from one strain to the other; *b*) no segregation at all, all spores alike and identical to the parent strain, if perithecia were formed by a single strain under hormonal influence of the other; *c*) segregation of sex factors in Mendelian ratio but conidial character always that of the " sole " parent if the gene for sex were diffusible. In the analysis of about 50 asci the first situation obtained for each ascus, proving conclusively that the distance between the two ends of the tube had been bridged by hyphal growth and that compatible nuclei had been brought together. The same results were again obtained when the work was repeated using the same strains as used previously by Moreau. She also disputed Moreau's further claim (124) that two mycelia of the same compatibility type grown together on a single plate produce perithecia. In many trials her results were uniformly negative.

A final paper by Moreau, in 1938 (121), cited new " evidence " for hormonal activity in *Neurospora*. The mycelium of one sexual strain, growing on a plate of agar, was heated for ten minutes in steam at atmospheric pressure to kill the vegetative thallus. Agar blocks, one centimeter square, were cut from this plate and placed on the surface of a living mycelium of the compatible strain. Perithecia were formed in a certain percentage (not cited!) of the

cases. If the temperature was carried as high as 110° C for ten minutes, no perithecia were formed, while if the temperature did not exceed 90 to 94° C, perithecia formed in every case. It was concluded from these results that hormones were present in the medium but that they were destroyed by a temperature very close to that required to kill the thallus. No evidence was given that all of the thallus was actually dead, and no genetical analysis of sex or conidial characters of the progeny from such perithecia was made. In the absence of such evidence the work must be considered with some suspicion.

Lindegren (91, 92) claims to have confirmed the existence of hormones in *Neurospora* as described by Moreau and Moruzi: "The writer (1934*a*) has confirmed the existence of hormones in *Neurospora.* A bisexual, heterokaryotic, self-sterile strain (containing f+ and f− nuclei which were incapable of copulation because of sterility factors) was mated to a highly fertile mutant strain (F+). Two kinds of zygotes resulted: (1) f+/f− and (2) F+/f−. Hormones from the F strain had enabled the sterile strains to copulate. Moreau and Moruzi assumed that their wild strain was homokaryotic and that hormones had stimulated it to reproduce parthenogenetically. It was probably bisexual and self-sterile and the hormones probably supplied an essential substance".

Lindegren's double admission of uncertainty, necessitating two "probably"s in the statement integrating his own observations with the experimental findings of the French workers, would appear effectively to reduce his "confirmation" to the status of a personal opinion. In the tremendously complicated situation arising in the intimate association and mating of two strains, involving three kinds of nuclei, hormones might well be expected to be involved, but in the total absence of other evidence, the reaction described above in no way constitutes proof or serious evidence of diffusible chemical regulators of any stage in the sexual process.

Two further reports of chemical regulation of restricted stages in the sexual processes of members of the Sphaeriales make less extensive claims than those cited above and are based on far sounder observational and experimental evidence.

Backus (9), in working out the mechanics of ascogonial fertilization in *Neurospora sitophila,* observed two phenomena which indicated hormonal activities: *a*) germination of conidia of one

race was observed to be completely inhibited in regions previously overgrown by the mycelium of the opposed compatibility strain; this effect obtained either upon agar or glass surfaces and was interpreted as the specific effect of a strain-specific inhibiting substance, a product of the plant's metabolism; *b*) the long sparsely-branched trichogyne commonly produced no short lateral branches in the absence of conidia, microconidia or hyphal fragments of the opposed compatibility strain in the immediate vicinity; in the presence of such potential fertilizing bodies, however, one to several lateral branches were regularly observed to arise de novo upon the trichogyne and grow directly to the sources of the fertilizing nuclei.

A preliminary study designed to confirm and extend the first of these two observations of Backus was carried out upon *N. crassa* in this laboratory by H. P. Papazian. A small but significant inhibition of conidial germination was consistently found in the filtrates derived from vegetative cultures of the compatible strain as compared with filtrates from cultures of the same mating type. The inhibition was far from complete in any case, however, and the study was shortly abandoned.

Conclusive evidence has been given by Zickler (183) of hormonal activity in the development of the trichogyne of a related species, *Bombardia lunatus*. In a culture containing both mating types the trichogynes appeared to be strongly and positively chemotropic to masses of spermatia discharged from spermagonia. In a single spore culture, i.e., single mating type, the trichogynes grew straight, but upon addition of spermatia from a compatible strain to such a culture, each trichogyne initiated a characteristic sinuous growth and grew directly to the largest clump of spermatia in the vicinity. In subsequent experiments the chemical nature of the attracting agent was proved. The filtrate from a suspension of spermatia was shown to induce positive chemotropic growth of trichogynes, these latter growing directly toward and into the tip of a capillary filled with the filtrate and inserted into a region having numerous ♀ sexual apparatuses. Boiling had no deleterious effect upon the substance(s) secreted by the spermatia and responsible for the tropic attraction of growing trichogynes.

An interstrain effect superficially similar to that postulated earlier by Lindegren (92) for *Neurospora* has been reported by Markert (95) in *Glomerella* (*cingulata*?). Two weakly compati-

ble strains mated on agar but separated by means of a permeable membrane from a third strain, which was strongly compatible with one of the mates, produced a far greater number of perithecia than a mating of the same strains in the absence of the third strain.

An attempt by Hirsch (53) to demonstrate hormones, suspected of being involved in the expression of sexual and compatibility characteristics, in *Hyphomyces solani* f. *curcubitae* was unsuccessful.

SEXUAL HORMONES IN GAMETANGIAL COPULATION

Among the organisms characterized by gametangial copulation, unequivocal demonstration of the correlation of sexual processes by diffusible secretions has been accomplished only in the Mucorales of the Phycomycetes. The few observations indicating sexual hormones in certain Ascomycetes having this type of sexual fusion unfortunately have not been followed up, and no clear-cut demonstration of chemical regulation has yet appeared for this group of fungi.

The very first demonstration of the initiation and coordination of sexual reproductive processes by auto-secreted diffusible substances in any plant was given by Burgeff in 1924 for *Mucor mucedo* (23). In matings of (+) and (−) strains of this plant upon an agar medium, a " restraint area " was formed along the line of intermingling of the two mycelia, and into this region only a few hyphae penetrated from each mycelium. At the tips of these hyphae corraloid swellings developed before actual contact was established, and upon these there were produced numerous protuberances which appeared to be progametangia. These reactions were considered to be the initiation of the sexual process, and, since they occurred before any contact between (+) and (−) hyphae, they indicated the presence and activity of diffusible chemical secretions.

To test this hypothesis Burgeff performed the classical experiment of physically separating plants of (+) and (−) strains with a permeable collodion membrane, thus maintaining continuity of the liquid medium through the short distance between the two plants. This was accomplished by placing a membrane upon the surface of a mycelium of the (−) strain growing upon agar and then placing a block of agar containing (+) hyphae upon the membrane. As the (+) hyphae grew out of the block of inoculum and

spread fan-wise across the membrane, the characteristic corraloid swellings, the zygophores or sexual organ primordia developed beneath them on the (−) mycelium. Thus sexual activation at a distance by means of diffusible secretions of the plants themselves was proved. This response was termed " telomorphosis ". Also, in a number of cases, compatible hyphae on opposite sides of the membrane were mutually attracted; this he termed " zygotropism ". Other species, including *Rhizopus nigricans, Mucor hiemalis* and *Phycomyces nitens,* were tested in similar membrane matings, but no trans-membrane activation was observed between compatible strains of these species.

Blakeslee (16), early in his study of heterothallism in the Mucorales, had shown that two compatible mycelia of *R. nigricans,* growing upon bits of bread which were suspended in juxtapotition within a moist chamber, were sexually activated only in the region of intermingling and had concluded that sexual stimulation depended upon contact of vegetative elements of the two compatible mycelia. Burgeff, however, speaks of the transfer of sexuality-inducing substances and of zygotropic substances through the air in connection with *M. hiemalis, R. nigricans* and *P. nitens* as though this mode of activation and determination of growth patterns were a well established and familiar fact. He neither gives nor cites any experimental evidence for air-borne sexual activators, nor has a literature search by the author revealed any source of such evidence.

During the decade following publication of Burgeff's demonstration, other workers, including Verkaik (179), Kohler (64), Ronsdorf (157), Kehl (58) and Krafczyk (66, 67), investigated the mechanism of sexual activation and coordination in a number of other species of the Mucorales. The major findings of Burgeff were confirmed by these subsequent workers, and most of the phenomena observed by him in *M. mucedo* were extended to other species. In only one case, however, was any substantial addition made to the bare demonstration of sexual hormonal activity in these forms.

Krafczyk (66, 67) in an intensive study of the formation and germination of zygospores in *Pilobolus cystallinus,* was able to repeat the hormonal induction of sexual organ primordia and the mutual attraction of hyphae of compatible strains. He demon-

strated, moreover, that gametangia of both strains could be hormonally induced without physical contact between any portions of the two mycelia. Through careful correlation of certain basic experimental results and detailed examination of the morphological progression in the sexual reaction, he was able to advance the convincing hypothesis that initiation and coordination of the sexual reaction were wholly under hormonal control until differentiation of the gametangia. In contrast to previous similar studies on other members of the Mucorales, his results showed clearly that each of the interacting mycelia both secreted sexual hormones and reacted to the secretions of its mate. His experimentation, unfortunately, was neither specifically designed nor sufficiently extended to yield the detailed information necessary to define the correlative mechanism in terms of specific substances inducing specific responses. Such definitive studies in the Mucorales remain to be made.

Although sexual hormones have not been demonstrated in ascomycetous fungi characterized by gametangial copulation, circumstantial evidence of such regulatory secretions has been furnished in one case.

An inquiry of Dodge (32) on the sexuality and sexual mechanism of *Ascobolus magnificus* brought out two interesting facts as regards the possible chemical regulation of sexual processes: *a*) Antheridia and ascogonia are never formed on monosporous mycelia but only when two compatible strains are grown together, the male and female organs invariably originating from different hyphae. He was unable, however, to determine whether they arise from different mycelia as well. *b*) The ascogonium develops beyond the primordial stage only if the trichogyne remains in contact with the antheridium. The author assumed a chemical mechanism to explain these phenomena. In a later paper (174) the hypothesis was stated as follows: " In all probability the gametophytes [of *A. magnificus*] come to maturity and produce their reproductive structures only when grown together in the same medium, the mycelium of each sex so changing the nature of the medium as to stimulate the development of the reproductive structures on the mycelium of the opposite sex ". The seemingly short step to recognition of the possibility of the secretion and interaction of specific sexual stimulatory substances was surprisingly not made.

Ascobolus magnificus appears to be unique among the many

intensively studied hermaphroditic self-sterile fungi (e.g., the eight-spored species of *Neurospora, Sordaria anserina,* the heterothallic rusts) in that sexual organs develop only after the two compatible mycelia are in actual contact. This behavior strongly suggests the activity of diffusible sexual hormones in the formation of sexual organs similar to those which have been demonstrated in the heterothallic members of the Saprolegniales and Mucorales. Of all species of Ascomycetes in which the life histories have been thoroughly investigated, *A. magnificus* holds the greatest promise for detailed elucidation of a sexual hormonal mechanism in this large class of fungi.

SEXUAL HORMONES IN SOMATIC COPULATION

Sexual hormones per se have not been demonstrated in any species characterized by somatic copulation. This type of copulation, in plants lacking both differentiated sexual organs and gametes, accomplishes the basic requirements of sexuality by conjugation of vegetative cells followed by reciprocal exchange of nuclei. Somatic copulation is the typical method of sexual interaction of the Basidiomycetes with the exception of the rusts. Although specific sexual regulators have not been demonstrated in these forms, the possibility of hormonal regulation of conjugative processes was early recognized and for more than a half century has remained a matter of considerable interest.

Following closely upon promulgation of the chemical theory of developmental regulation in plants by Sachs in 1880, a number of students of lower plants pointed out examples of correlative phenomena which might be explained by diffusible chemical regulators. Simultaneous formation of conjugation tubes and their subsequent apparent mutual attraction in the sexual reproductive process of the Conjugales, and fusion of sporidia of certain smuts by means of conjugation tubes, could be explained by such a mechanism (19, 130). A few years later the hypothesis was advanced by Ward, in 1888 (180), that such effects could be explained by secretion of cellulose-softening ferments from localized portions of the thallus, most likely by the elongating apices. These, by rendering the cell wall plastic, would allow internal cell pressure both to initiate tube development and to determine its directional growth along the concentration gradient. This hypothesis, while based on observations

of non-sexual hyphal fusions in *Botrytis,* could apply equally well to sexual or asexual processes involving tube formation.

Buller (22), in Vol. V of his Researches on Fungi, devotes an entire chapter to the subject of hyphal fusions and reviews in considerable detail the long history of this problem. He pointed out that, in addition to the absence of any experimental evidence as to the nature of the correlative mechanism, no theoretically feasible hypothesis has so far been advanced to explain the remarkably precise progression of events in the mutual and tropic attraction and fusion of closely associated hyphae. A basic and common type of mechanism appears to coordinate hyphal anastomoses throughout a majority of the septate fungi, regardless of *a*) the origin of the two hyphae or cells involved, whether originating on the same mycelium, on different mycelia of the same compatibility type, on different mycelia of different mating types, etc.; and *b*) the nature of the product resulting from the union, whether a sterile heterokaryon, a fertile heterokaryon, a stable dicaryon, or perhaps in most cases, involving no exchange of nuclei.

Buller pointed out the essential difficulty of hyphal-specific chemotropic regulators, but, in considering other possible mechanisms which might explain the observed facts, he overlooked the antithetical proposition: a single species-specific substance, labile and secreted in small quantities by every growing hyphal tip. Such a single substance mechanism, providing a steep concentration gradient of a chemotropic agent in the immediate vicinity of each hyphal tip, would result in a slight but possibly critical concentration gradient across each of two hyphae when they happen to lie within a very short distance of each other. Experimental evidence for such a mechanism is entirely lacking, and, since the agents responsible are known to be effective only through distances of the order of 10 μ, it is difficult to imagine experimental procedures which would adequately test the hypothesis. A more likely though indirect approach might be made by way of detailed analysis of the temporal, spatial and angular relationships between hyphae during the period of tropic curvature and fusion.

Aside from the unsolved puzzle of hyphal fusions, evidence for hormones is almost entirely wanting in plants which show somatic copulation. Harder (43), in connection with microsurgical isolation of the two component nuclei of the dicaryon of the Hymeno-

mycete, *Schizophyllum commune*, suggested, without any concrete evidence, the hormonal control of clamp connection and pseudo-clamp formation.

SUMMARY AND CONCLUSIONS

Convincing evidence for the chemical control of sexual processes in plants, a concept first advanced by Sachs in 1870, has accumulated slowly during the past half-century. At the present time our knowledge of the role of chemical regulators of sexual phenomena in plants pertains almost exclusively to species belonging to the Thallophytes. The promising exception to this generalization is, of course, the gradually unfolding story of the floral initiating substance(s) in flowering plants.

The rationale of the greater advances in this field in work with forms which are commonly conceded to be among the more primitive of plants may follow from either, both or neither of the two following possibilities: *a*) relative simplicity of the basic processes in the lower plants, and *b*) the relatively greater influence of external agents upon such processes. One observation is of interest in this connection: the two forms for which integrated chemical correlative mechanisms have so far been defined, *Chlamydomonas* and *Achlya*, belong to the more primitive groups of the algae and fungi, respectively, and both of them are strictly aquatic plants. The continuously aquatic habitat of these forms, however, may be inconsequential, since few, if any, algae or fungi can dispense with an effectively aqueous environment for that portion of their developmental histories during which sexual processes occur.

Two categories of chemical regulation of sexual manifestations can be recognized: *a*) regulation by chemical agents of extra-specific origin; *b*) regulation by agents of intra-specific origin.

The first category includes those cases which show marked influence, either stimulatory or inhibitory, of chemical substances of extra-specific origin upon the final sexual productivity of the plant. Chemical regulation of this type has been described to date only in the fungi. The active compounds, because of their external origin and their hormone-like activities, have been designated "ectohormoids". The source of the active compound is incidental, but in most of the recorded cases they have been shown to be metabolic products of other plants, chiefly bacteria and other fungi. Among

the compounds producing effects of this type are a number of organic acids, various members of the vitamin-B complex, a few substances having physiological activity in mammals such as histamine and oestrin, etc. The effects of these chemical substances upon the sexual reaction is seldom critical; an external supply of the active agent is rarely indispensable for normal progression or consummation of the sexual process. It is possible that those cases characterized by marked sexual stimulation reflect sub-optimal production of required substances by the affected plants' own metabolic processes, whereas the few known cases of marked inhibition of sexual productivity would appear most easily explicable as physiological accidents. In either case it appears unlikely that regulation of sexual processes by chemical agents of external origin has played any decisive role in the evolutionary history of the fungi.

The second category comprises those cases showing the activity of specific substances secreted by the plant itself and which are essential for the initiation and coordination of the sexual process, or stages thereof, in the individual or between individuals of the same species. Such substances, performing indispensable roles in a vital process of the plant's developmental history, may truly be considered and termed " sexual hormones ". The activities of the sexual hormones are, in almost every case, extremely specific, each hormone or complex of two or more hormones initiating and regulating a characteristic stage of the sexual progression. This characteristic of sexual hormones further distinguishes them from ectohormoids which appear to affect all stages of the sexual response, probably through an effect upon the over-all vigor of the plant.

Following Burgeff's pioneering demonstration in 1924 of sexual hormones in *Mucor mucedo,* chemical regulation of sexual processes by specific auto-secreted hormones have been found in all of the major groups of algae and fungi with the exception of the Basidiomycetes and the Rhodophyta. There is no convincing evidence that there exists a single basic mechanism common to a majority of the members of the Thallophytes; on the other hand, the knowledge of hormonal control is spotty and as yet of insufficient scope to refute the possibility of such a common basic pattern.

The distribution of the demonstrated cases is most easily comprehended in terms of the four basic types of sexual mechanism to be found among the Thallophytes.

GAMETIC COPULATION. Clear evidence of sexual hormones has been found in species of all major groups of algae reproducing by planogametes. The more important of these are the genera: *Chlamydomonas, Protosiphon, Hydrodicton, Cladophora, Dasycladus* and *Acetabularia* of the Chlorophyta, *Fucus* and *Ectocarpus* of the Phaeophyta, and *Botrydium* of the Chrysophyta. In all of these forms the hormones are secreted by and elicit one or more specific responses in the gametes themselves, as follows: flagellar formation, motility, sexual determination, chemotaxis, copulation. Because of the striking similarity of gametic morphology and behavior among the algae which reproduce by planogametes, it appears not unlikely that they possess a common coordinative mechanism; the negative results in the few instances in which the hormones of one form have been tested with other forms, however, would indicate that hormonal specificities prevent interaction between all but very closely related species.

A different type of sexual regulation has been found in *Monostroma,* of the Ulvaceae. In this form a constituent of the gametophytic thallus has been shown to affect the differentiation of the swarmers produced by the sporophyte as gametes instead of as zoospores; the gametophytic generation, except the gametes themselves, may thus be by-passed experimentally.

No sexual hormonal activity has been reported in those fungi which reproduce by gametic copulation.

GAMETE-GAMETANGIAL COPULATION. In plants characterized by gamete-gametangial sexual apparatuses, sexual hormones have been reported in a number of species belonging to two groups of fungi, the Saprolegniales of the Phycomycetes and the Sphaeriales of the Ascomycetes. In *Achlya,* of the Saprolegniales, hormones have been shown to initiate and coordinate the entire sexual process, involving principally formation and differentiation of the sexual organs. Clear demonstration of hormonal activity in the Sphaeriales has to date been limited to the directed growth of trichogynes to masses of extruded spermatia in *Bombardia;* other reports indicate similar activity in *Neurospora.*

GAMETANGIAL COPULATION. Only in the phycomycetous order, Mucorales, has unequivocal proof of the activity of sexual hormones been given for plants characterized by gametangial fusion. Production of sexual organs and tropic responses between elements

of compatible mycelia have been shown to result from the activity of diffusible hormones in numerous species of this order, including *Mucor mucedo,* the plant used in Burgeff's original demonstration of sexual hormones, various other species of *Mucor, Phycomyces, Pilobolus,* etc. For *Pilolobus* there is indicative, though not conclusive, evidence that the sexual reaction is under hormonal control until the gametangia are differentiated.

SOMATIC COPULATION. Sexual hormones have not been demonstrated in plants which show somatic copulation. Chemical agents have long been suspected of inducing and coordinating the process of hyphal fusion but experimental evidence for such activity is completely lacking.

In only two of these many cases in which sexual hormones have been reported has work been pursued to the point where it has been possible to describe the over-all mechanisms responsible for the correlation of sexual phenomena, i.e., the probable number of specific substances involved, the conditions under which they are secreted, the specific responses which they induce, etc. These two investigations are the works of Moewus, Kuhn, et al. on the unicellular green alga, *Chlamydomonas,* and that of the author on the filamentous aquatic fungus, *Achlya.* Each of these two studies shows the respective sexual correlative mechanism to be a complex of many successive stages, the several stages being chemically interdependent as regards their initiation and control. Otherwise there appears to be but little similarity between the two mechanisms. It should be recalled, however, that the sexual processes of the two plants are quite dissimilar, one reproducing by gametic copulation, the other by gamete-gametangial copulation. Furthermore, the stages of the sexual reproductive process studied in the two cases are not strictly comparable: the mechanism described for *Achlya* pertains to the formation and coordinated development of the sexual organs up to the time of differentiation of the ♀ gametes, or eggs, whereas that in *Chlamydomonas,* in which each vegetative cell can function as a gamete, deals almost exclusively with the reactions of the gametes themselves.

The investigation on *Chlamydomonas* is quite unique in that, of all studies on sexual regulatory phenomena in the Thallophytes, it alone has approached the ultimate objective of integration of the sexual mechanism with the other basic activities of the plant. The

several hormones have been identified, the biochemical sequences leading to elaboration of the hormones have been determined, and the genetics of the organism, including specific genic control of most of the reactions leading to production of the sexual hormones, have been minutely elucidated. The work, unfortunately, has been only partially confirmed by independent investigators, chiefly as regards purely qualitative physiological aspects, while there is no confirmation of the biochemical and genetic aspects. Internal discrepancies in the work itself and this lack of confirmation, particularly in view of numerous unsuccessful attempts by other workers to repeat, in closely related species, essential portions of the work, have resulted in widespread scepticism of the validity of the work, in part or in its entirety.

Further elucidation of chemical regulation of sexual processes in the Thallophytes constitutes a challenging problem—a problem which is open for ready exploitation. The accomplishments which have been made up to the present time indicate the intriguing possibilities of the field and serve as signposts pointing to the more promising areas for future endeavors.

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